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14. ABSTRACT Mutations in the p53 tumor suppressor gene are the most prevalent genetic events in human Her2-positive breast cancer and are associated with poor prognosis. Frequently in early stages of cancer, a p53 mutation in one allele is followed by loss of heterozygosity (LOH) in the second allele, during tumor progression. Despite a strong notion that p53 mutations with subsequent LOH are driving events in breast cancer, the translational significance of p53 mutational and LOH status, and their role in breast cancer development and progression have not been evaluated. Previously we found that the irradiation of pre-malignant lesions of p53 heterozygous mice (R172H p53 mutant or null) aggravates tumorigenesis in an MMTV/ErbB2 mouse breast cancer model. This study aims to test whether spontaneous or genotoxic stress-induced LOH promotes progression in mutp53 heterozygous tumors and whether different hot-spot p53 mutations are equal in this respect. The major innovative findings for reporting period: we generated and analyzed novel R248Q/+;ErbB2 mouse model. We found that in initially heterozygous mouse tumors carrying the R248Q (p53Q/+) allele, the loss of the remaining wild-type p53 allele is necessary prerequisite for mutant p53 stabilization and gain-of-function (GOF) in vivo. In mouse tumors with high frequency of p53 LOH (osteosarcomas and fibrosarcomas), we find that mutant p53 protein is stabilized (94%) and tumor onset is significantly accelerated compared with p53+/- tumors. In contrast, in mouse tumors with low frequency of p53 LOH (MMTV-Neu breast carcinomas), mutant p53 protein is not stabilized (80%) and GOF is not observed. Thus, p53 LOH is a critical prerequisite for missense mutant p53 stabilization and GOF in vivo.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	11
5.Changes/Problems.....	12
6. Products.....	13
7. Participants & Other Collaborating Organizations.....	14
8. Special Reporting Requirements.....	16
9. Appendices.....	17

1. INTRODUCTION:

Mutations in the p53 tumor suppressor gene are the most prevalent genetic events in human Her2-positive breast cancer and are associated with poor prognosis and survival. Frequently in early stages of cancer, a p53 mutation in one allele is followed by loss of heterozygosity (LOH) in the second allele, during tumor progression. And despite a strong notion that p53 mutations with subsequent LOH are driving events in breast cancer, the translational significance of p53 mutational and LOH status, and their role in breast cancer development and progression have not been comprehensively evaluated, especially in the context of conventional genotoxic modalities. Previously we found that the heterozygous mutp53 R172H allele increased the frequency of p53 LOH in mammary tumors compared with the p53- null allele, which correlated with aggravated tumorigenesis in an MMTV/ErbB2 mouse breast cancer model. This phenotype became even more prominent after γ -irradiation of mice with premalignant lesions, which led to a dramatic increase in metastases in the presence of mutp53 allele. These data strongly suggest that DNA damage further augments the oncogenic activity of mutp53. Thus, we hypothesize that at early stages of breast cancer, genotoxic therapies in the long run can promote tumor progression in mutp53 heterozygous tumors by (1) inducing the loss of the remaining wtp53 allele and thus, its tumor suppressor activity; (2) stabilizing mutp53 protein over the threshold needed to manifest its full oncogenic potential; (3) p53 LOH-mediated acquisition of metastatic properties; (4) amplifying ErbB2 and its related HSF1 (Heat Shock Factor1) signaling. This proposal aims to evaluate the physiological consequences of p53 LOH in breast cancer initiation, progression and metastases and assess how mutp53 affects response to genotoxic therapies with regard to p53 LOH status; how the response changes at different stages of breast cancer; and the short- and long-term therapeutic effects of genotoxic treatments.

2. KEYWORDS: p53, mutant p53, ErbB2, Her2, breast cancer, loss of heterozygosity (LOH), radiation, chemotherapy, Her2 positive breast cancer.

3. ACCOMPLISHMENTS:

The major goals of the project.

Major Task 1. Determine the effect of DNA-damaging therapeutics on p53 LOH and tumorigenesis in ErbB2-driven mutp53 mammary tumors *in vivo*.

Subtask 1. Define the physiological consequences of p53 LOH in ErbB2-driven mammary tumorigenesis. Analyze histopathology, the ErbB2/HSF1 signaling by IHC and Western in the established collection of mammary tumors from irradiated and non-irradiated mice with different p53 LOH status. (timeline months: 1-12, 50% completion).

Subtask 2. Evaluate the effect of different p53 mutations on p53 LOH in ErbB2-driven mammary tumorigenesis. Test whether similar to R172H, R248Q mutant p53 allele aggravates mammary tumorigenesis compared to p53 null counterparts and promotes p53 LOH after irradiation. (timeline months:1-24, 50% completion)

Subtask 3. Assess the effect of irradiation of established mutp53;ErbB2 tumors on p53 LOH (neoadjuvant setting). Test whether irradiation of established tumors induces LOH and accelerates mammary tumorigenesis in R172H/+;ErbB2 mice.

First, we will expand our breeders colonies to generate 60 females of each genotypes: p53-/+;ErbB2, H/+;ErbB2 and +/+;ErbB2. (timeline: months 1-12, 80% completion).

30 mice of each genotypes will irradiated with single dose of 5Gy irradiation at the time of tumor presentation.

The monitoring and analysis as described for the Aim1b. 60 females p53-/+;ErbB2 + 60 females H/+;ErbB2 60 females +/+;ErbB2 = 180 total animals. (timeline: months 12-32, 0% completion).

Subtask 4. Determine whether generic genotoxic drug doxorubicin promotes p53 LOH in established R172H/+;ErbB2 tumors in neoadjuvant setting.

To test whether commonly used for Her2 positive breast cancer treatment genotoxic drug doxorubicin similar to irradiation induces LOH in mutp53 dependent manner, 30 H/+;ErbB2 females, 30 p53-/+;ErbB2 and 30 +/+;ErbB2 females will be treated with 4mg/kg doxorubicin (dox) in PBS intraperitoneally at the time of tumor onset (0.5 cm³, volume) once daily for 5 consecutive days. Monitoring and analysis will be performed as described in Aim 1b. 60 females p53-/+;ErbB2 + 60 females H/+;ErbB2 + 60 females +/+;ErbB2 = 180 total animals (timeline: months 12-32, 0% completion).

Local IRB/IACUC approval (100% completion)

HRPO/ACURO approval (100% completion)

Major Task 2. Mechanistically assess the physiological consequences of p53 LOH in heterozygous mutp53 mammary cells *in vitro*.

Subtask 1. Examine the frequency and time of p53 LOH onset in existing collection of cell culture of primary mammary epithelial cells and mammary tumors culture derived from mice with different p53 genotypes. Serial passaging of R172H/+ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells. (timeline: months 12-24, 20% completion).

Subtask 2. Test the effect of irradiation on the frequency and time of p53 LOH onset in primary mammary epithelial cells (MECs) and mammary tumors culture derived from mice with different p53 genotypes. Serial passaging of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells after single dose of irradiation *in vitro* at passage 1. (timeline: months 24-32, 20% completion).

Subtask 3. Correlate the p53 LOH status of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells with cellular properties (proliferation, chemoresistance, allografts) and with biochemical characteristics (ErbB2 and HSF1 signaling). (timeline: months 24-32, 0% completion).

Major Task 3. Determine whether p53 LOH promotes metastatic behavior in ErbB2 cancer cells.

Subtask 1. Establish whether p53 LOH enhances the motility and invasion of cancer cells *in vitro*.

Test the motility and invasive properties of primary mammary epithelial cells and tumor cultures derived from H/+;ErbB2 and p53-/+;ErbB2 mice before and after LOH *in vitro*. Boyden chamber assay, wound healing assay, metastases in allografts. (timeline: months 24-32, 0% completion).

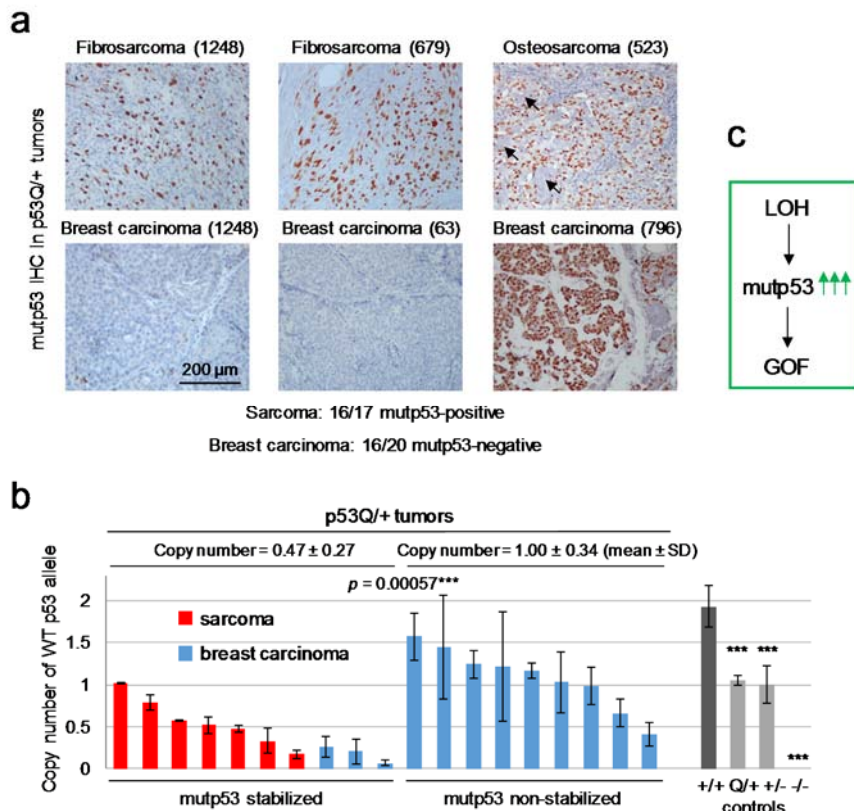
Subtask 2. Determine whether p53 LOH enhances the ability of tumor cells to metastasize *in vivo*. Isolate metastatic cells from lungs of irradiated and non-irradiated of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 mice. Assess p53 LOH status in metastases in comparison with primary tumors. (timeline: months 24-32, 0% completion).

The major accomplishments of the project up-to-date

Major Task 1. Determine the effect of DNA-damaging therapeutics on p53 LOH and tumorigenesis in ErbB2-driven mutp53 mammary tumors *in vivo*.

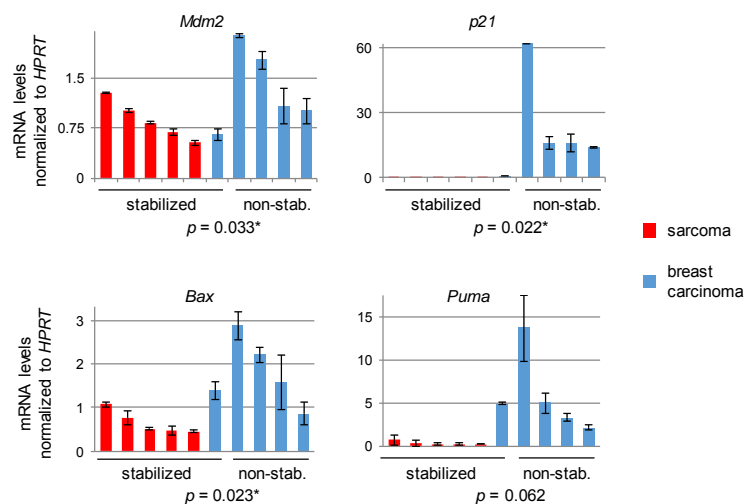
Subtask 1. Define the physiological consequences of p53 LOH in ErbB2-driven mammary tumorigenesis. Analyze histopathology, the ErbB2/HSF1 signaling by IHC and Western in the established collection of mammary tumors from irradiated and non-irradiated mice with different p53 LOH status.

The translational significance of tumor-specific stabilization of mutant p53 was established previously. The purpose of this subtask is to 1) comprehensively evaluate mutant p53 protein levels in mammary tumors with regard to p53 LOH status; 2) correlate the mutant p53 levels/LOH status with physiological outcomes.



First, we evaluated consequences of spontaneous LOH that occurs in the absence of genotoxic treatments. We analyzed histopathology and p53 IHC staining in non-irradiated tumors with the respect to p53 LOH status. For these experiments we utilized newly generated R248Q;Neu mouse model. We crossed the heterozygous breast cancer hotspot mutant p53 allele R248Q ('p53Q/+') with the MMTV-Neu ('Neu') mice expressing additional wild-type ErbB2 copies selectively in the mammary gland. Surprisingly, histopathological analysis revealed that about half of p53Q/+;Neu and p53-/-;Neu mice did not develop breast cancer but instead developed osteosarcomas and fibrosarcomas, which originate from mesenchymal tissues where MMTV-Neu is not expressed. This data suggests, that presence of one mutp53 Q allele is sufficient to drive tumorigenesis even in the absence of

Fig.1 Loss of wtp53 allele is required for missense mutant p53 stabilization and GOF. (a) The vast majority of sarcomas (16/17 cases, 94%) have stabilized mutp53. In contrast, the majority of breast carcinomas (16/20 cases, 80%) do not. Immunohistochemistry for mutp53. Mouse identity in parentheses. Arrows indicate the osteoid in osteosarcoma. (b) Analysis of wtp53 copy number in sarcomas and breast carcinomas of p53Q/+;Neu mice by quantitative genotyping. Tumors with mutp53 stabilization (all sarcomas and three breast cancers tested) have significantly higher LOH than tumors without mutp53 stabilization (majority of breast cancers). Note, as sarcomas have highnormal stroma contamination (top, blue mutp53-negative stromal cells, which do not have LOH), the actual LOH in sarcomas is most likely even higher because of dilution of the tumor genotype, causing LOH underestimation. For the same reason, copy numbers of the two highest sarcoma cases (two left red bars) are likely inflated. The wtp53 signal was normalized to the Rosa26 signal. Tail biopsies from p53+/+ (two wt alleles), p53Q/+, p53-/+ (one wt allele) and p53-/- mice (no wt alleles) were used as normal control tissues without LOH. Bars represent mean \pm S.D. of two technical replicas of individual cases. ***Po.001. (c) Schematic diagram of the proposed mechanism for mutp53 stabilization and GOF in heterozygous tumors. Loss of the wtp53 allele (LOH) causes accumulation of highly stabilized mutp53 protein, which triggers tumor development and is the principle mechanism and prerequisite of GOF



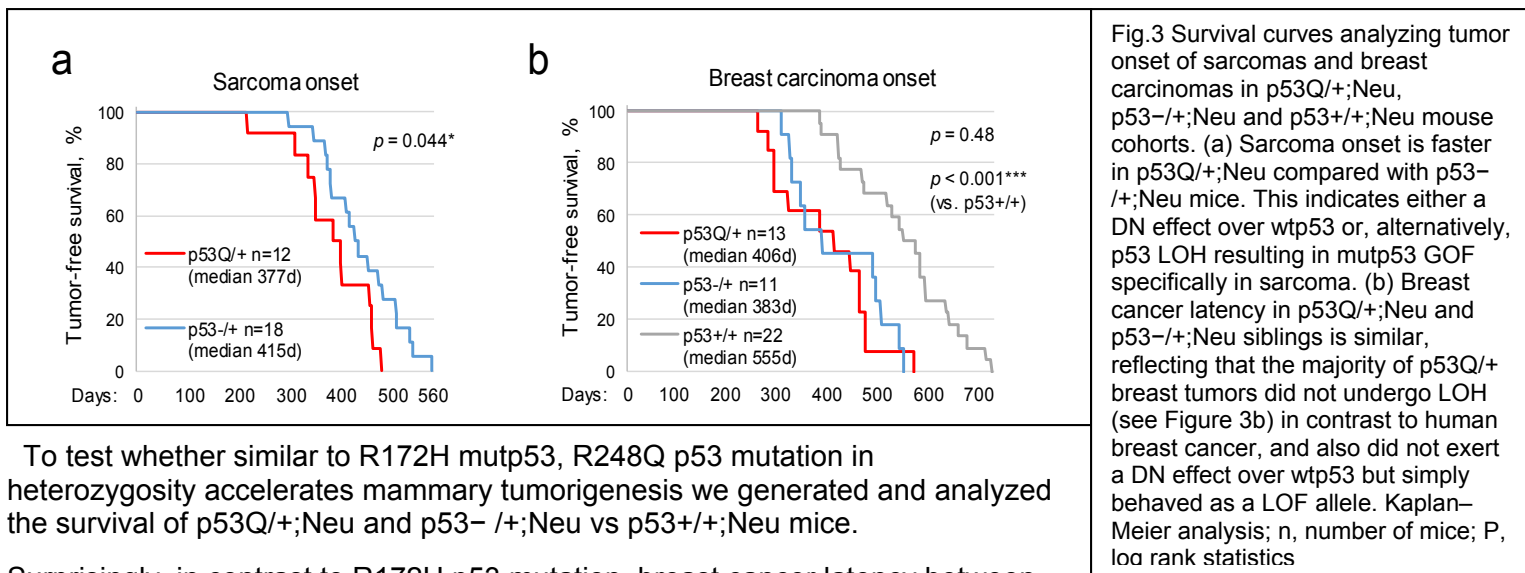
ErbB2 in tissues of the mesenchymal origin. Strikingly, IHC analysis of tumors revealed mutp53 stabilization in nearly all examined sarcomas (94%, 16/17), but only in rare breast carcinomas (20%, 4/20), even within the same animal (Figure 1a, e.g., animal #1248). Our previous work has established that high level of mutant p53 in cancer cells is essential for its oncogenic activity. Thus, we asked whether mutant p53 stabilization in sarcomas is the result of wtp53LOH and whether sarcomas are more prone to p53 LOH than breast tumors. Indeed, qPCR of genomic DNA showed that p53

Fig.2 Real-time qPCR analysis of wtp53 target genes Mdm2, p21, Bax and Puma shows that their expression is largely decreased in samples with mutp53 stabilization

spontaneous LOH occurs in all sarcomas, but less frequent in breast cancer (Figure 1b). Moreover, the few breast tumors that did stabilize mutp53 also underwent p53 spontaneous LOH. Together, this strongly suggests that LOH is a critical prerequisite for mutp53 stabilization in cancer cells (Figure 1c). Importantly, LOH and its related stabilization of mutant p53 protein strongly correlate with the tumor onset and survival. We observed oncogenic effect of mutant p53 only in sarcomas, where LOH and stabilization of mutant p53 are more profound than in breast carcinomas. Sarcoma onset is faster in p53Q/+;Neu compared with p53-/-/+;Neu mice (Figure 3a).

The stability of mutant and wt p53 is maintained by Mdm2, p53-specific E3 ligase, which itself is the transcriptional target of wtp53. Hence, the low level of mutant p53 protein in heterozygous mammary tumors, most likely, is attributed by transcriptional activity of wtp53. There is a strong notion in the field, that in heterozygosity mutant p53 inactivates wtp53 via dominant-negative mechanism. To test this idea in our model, we analyzed p53 target genes in tumors as readout for the remaining wtp53 allele activity (Figure 2). Indeed, all tumors with stabilized mutp53, including the single 'outlier' breast cancer tested, had reduced or undetectable Mdm2 and p21 levels, respectively, and sarcomas also had reduced Bax and Puma expression correlating with their LOH. Hence, our data suggests that in heterozygosity wt p53 retains its transcriptional activity and its tumor-suppressive function. While, induced and/or spontaneous LOH would lead to complete loss of wtp53 function, manifestation of mutant p53 gain-of-function (GOF) and, thus, augment tumor progression. We will test this hypothesis in our further experiments. Next, we will evaluate the effect of spontaneous LOH on ErbB2/HSF1 axis in vivo IHC analysis of mammary tumors with/without LOH.

Subtask 2. Evaluate the effect of different p53 mutations on p53 LOH in ErbB2-driven mammary tumorigenesis. Test whether similar to R172H, R248Q mutant p53 allele aggravates mammary tumorigenesis compared to p53 null counterparts and promotes p53 LOH after irradiation.

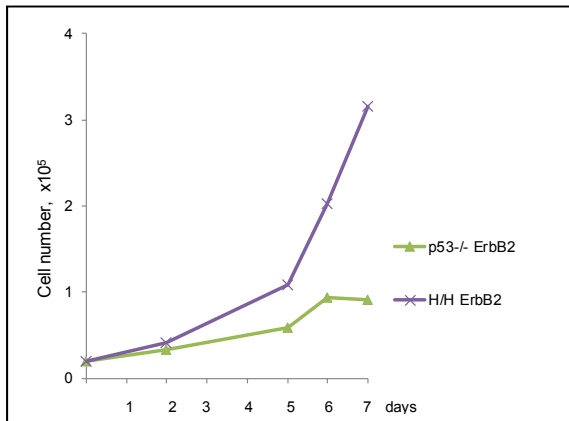


To test whether similar to R172H mutp53, R248Q p53 mutation in heterozygosity accelerates mammary tumorigenesis we generated and analyzed the survival of p53Q/+;Neu and p53-/-/+;Neu vs p53+/+;Neu mice.

Surprisingly, in contrast to R172H p53 mutation, breast cancer latency between p53Q/+;Neu and p53-/-/+;Neu siblings was similar (Figure 3), suggesting p53 mutation-specific effects on mammary cancer development and progression in ErbB2 context. Nevertheless, loss or mutation of one wtp53 allele dramatically accelerated mammary tumorigenesis suggesting strong wtp53 loss-of-function effect. Importantly, we found that the rate of spontaneous LOH is similar in both mouse models: H/+;ErbB2 mice (17%) and Q/+;ErbB2 mice (20%). Currently, we are continuing breeding and expansion of mouse colonies to further test whether similarly to R172H p53 mutation R248Q mutation augments LOH rate in response to genotoxic treatments (irradiation and chemotherapies).

Major Task 2. Mechanistically assess the physiological consequences of p53 LOH in heterozygous mutp53 mammary cells *in vitro*.

Subtask 1. Examine the frequency and time of p53 LOH onset in existing collection of cell culture of primary mammary epithelial cells (MECs) and mammary tumors culture derived from mice with different p53 genotypes. Serial passaging of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells.



Previously, we successfully established and passaged primary cultures of MECs derived from mammary epithelial of mice of following genotypes: H/H;ErbB2, -/-;ErbB2 and Q/-;ErbB2 (Figure 4). Although, H/H;ErbB2 and -/-;ErbB2 MECs proliferate at different rate, we were able to passage them indefinitely. Unexpectedly, in contrast to primary mouse embryo fibroblasts (MEFs) (Shetzer, Y. et al. The onset of p53 loss of heterozygosity is differentially

Fig.4 Mutant p53 promotes mammary cell proliferation. A, to measure cell growth rates, MECs of different genotypes were plated at day 0 and counted at indicated time points. Representative graph from two independent experiments

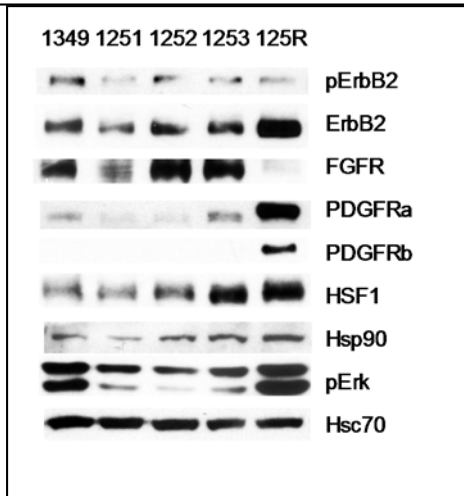


Fig.5 Biochemical characterization of ErbB2/HSF1 signaling in H/+;ErbB2 cancer cell lines established from mammary tumors of mice with the corresponding genotype.

induced in various stem cell types and may involve the loss of either allele. Cell death and differentiation 21, 1419-1431) we failed to passage MECs from H/+;ErbB2, -/+;ErbB2 and +/+;ErbB2 mice. All wtp53 expressing MECs undergo senescence following passage 3. This data is consistent with our observations that wtp53 in heterozygosity is able to exert its tumor suppressive function by inducing the transcription of subset of target genes. Therefore, now we mainly will focus on cell lines established from mammary tumors of H/+;ErbB2 (Figure 5), -/+;ErbB2 and +/+;ErbB2 cells. We successfully established mammary tumor cell lines from different H/+;ErbB2, -/+;ErbB2 and +/+;ErbB2 mice. First, we verified the genotype of established H/+;ErbB2 and -/+;ErbB2 cell lines – all of them still retain wtp53 allele. Our initial analysis has shown, that tumor cell lines in contrast to wtp53 expressing MECs can be propagated for indefinite time, even in the presence of wt p53 allele. H/+;ErbB2 tumor cells express high levels of ErbB2 and activated pErbB2, and pErk as readout of ErbB2 signaling. Murine cell lines show detectable levels of HSF1 and its targets heat shock proteins (Hsp90) (Figure 5). As expected, and consistent with the presence of wtp53 allele, we were unable to detect p53 protein levels, likely due to expression of p53 target- E3 ligase Mdm2. Thus, the initial biochemical

analysis of established cell lines identified functional ErbB2/HSF1 axis (Figure 5). Next, we will evaluate how spontaneous p53 LOH affects ErbB2/HSF1 signaling in vitro.

Our preliminary experiments indicated that passaging tumor cell lines leads to gradual loss of wtp53 allele in heterozygous cells. This observation raises the necessity for the quantitative assessment of LOH status in a cell population during passaging. To achieve accurate and quantitative assessment of wtp53 allele presence, we developed primers and optimized the protocol, which allow us to detect only wt or mutant p53 allele at once by quantitative PCR. Currently, using the optimized protocol we performing systematic quantitative analysis of passaged heterozygous cell lines with different genotypes (-/+;ErbB2 vs H/+;ErbB2). We are using the multiple biological replicas to definitively establish whether similarly to in vivo study LOH occurs more frequently in the presence of mutant p53 compare to p53 null heterozygote.

Subtask 2. Test the effect of irradiation on the frequency and time of p53 LOH onset in primary mammary epithelial cells (MECs) and mammary tumors culture derived from mice with different p53 genotypes. Serial

passaging of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells after single dose of irradiation in vitro at passage 1.

For the implementation of this subtask, we are performing series of experiments testing Her2 breast cancer conventional therapeutics: genotoxic (irradiation, camptotecin) vs non-genotoxic (taxanes, ErbB2-inhibitor lapatinib) to evaluate how different types of treatments affect p53 LOH status. As a first step, we ensured the efficacy, dose dependence (Figure 6) and specificity (Figure 7) of selected drugs, e.g. ErbB2 inhibition by lapatinib in murine cell lines. Similarly, we evaluated the toxicity of camptothecin (genotoxic compound) and docetaxel (disrupts the normal function of microtubules) in H/+;ErbB2 cell lines. Pilot quantitative PCR experiments using optimized protocol revealed 25% LOH in H/+;ErbB2 cells after one round of genotoxic treatment. Currently, we are continuing these experiment in multiple biological replicas of cell lines with different genotypes (-/+;ErbB2 vs H/+;ErbB2) and different drugs to recapitulate our in vivo results.

Subtask 3. Correlate the p53 LOH status of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells with cellular properties (proliferation, chemoresistance, allografts) and with biochemical characteristics (ErbB2 and HSF1 signaling).

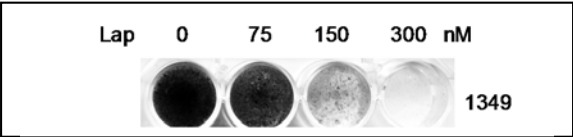


Fig. 6 Testing the sensitivity of H/+;ErbB2 murine cancer cells to lapatinib

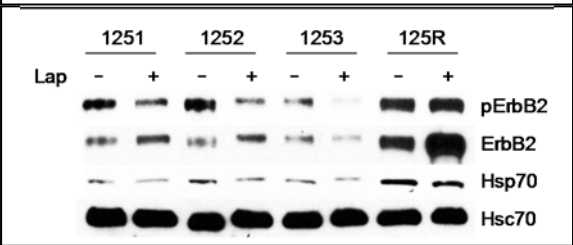


Fig. 7 Lapatinib inhibits ErbB2 signaling in H/+;ErbB2 cancer cell lines established from mammary tumors of mice with the corresponding genotype.

As mentioned above, our attempts to generate R172H/+;ErbB2, p53-/+;ErbB2, p53+/+;ErbB2 MECs failed due to premature senescence of wtp53 expressing MECs. Therefore, now we mainly will focus on mammary tumor cell lines with matching genotypes. After completion subtask 1 and 2 and generation cell lines, we will compare ErbB2, HSF1 and mutp53 signaling and phenotypical properties in cells from initial passages that retain wt p53 allele and homogeneous population of cells which underwent complete wtp53 LOH.

Major Task 3 Determine whether p53 LOH promotes metastatic behavior in ErbB2 cancer cells.

Subtask 1. Establish whether p53 LOH enhances the motility and invasion of cancer cells in vitro. Test the motility and invasive properties of primary mammary epithelial cells and tumor cultures derived from H/+;ErbB2 and p53-/+;ErbB2 mice before and after LOH in vitro. Boyden chamber assay, wound healing assay, metastases in allografts.

Subtask 2. Determine whether p53 LOH enhances the ability of tumor cells to metastasize in vivo. Isolate metastatic cells from lungs of irradiated and non-irradiated of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 mice. Assess p53 LOH status in metastases in comparison with primary tumors.

According to our SOW, the completion of Major Task 3 is contingent upon the accomplishment of Major Task 1 and 2. We expect to complete this subtask in year 2 and 3.

- What opportunities for training and professional development has the project provided?

Lucas Garcia, undergraduate student, Stony Brook University (September 2016-July 2017), Julia Rosenfeld, undergraduate student Binghamton University State University of New York, (June-July 2017) and Safia Mirza (high school student) have received professional on-hand training while working on this project. Lucas Garcia is the co-author on the manuscript submitted to Molecular Cancer Therapeutics. Partly as a result of this educational activity, Lucas Garcia was admitted to prestigious Graduate School of Boston Medical School.

- How were the results disseminated to communities of interest?

Nothing to Report.

- What do you plan to do during the next reporting period to accomplish the goals?

For the next reporting period we plan to test effect of different genotoxic vs non-genotoxic modalities on p53 LOH status in vivo and in vitro and test the physiological consequences of induced and spontaneous LOH depending on p53 genotype. We plan to utilize generated cohorts of mice with different p53 genotypes to assess effect of irradiation on established mammary tumors with regard to p53 LOH (neoadjuvant setting). Up-to-date we generated valuable preliminary data for the study, which will be completed within next 6 months and submitted as a manuscript to peer-viewed journal.

4. IMPACT

- Major innovative findings and achievements for this reporting period:

1) We completed generation and analysis of the novel mouse model for Her2 breast cancer R248Q;ErbB2. In contrast to previously generated R172H;ErbB2 mouse model, R248Q p53 mutation in heterozygosity does not accelerate mammary tumorigenesis. This data implies that cooperation of mutant p53 in ErbB2 occurs in p53 mutation type-specific manner.

2) The survival analysis of newly generated heterozygous R248Q;ErbB2 mouse model indicates strong loss-of-function, but not gain-of-function effect of mutant p53.

3) In addition to mammary tumors, significant fraction of R248Q;ErbB2 mice developed fibrosarcomas and osteosarcomas, which derived from tissues of origin which do not express ErbB2. This data indicates that in heterozygosity p53 mutation in one allele is sufficient to drive tumorigenesis.

4) The rate of spontaneous LOH in breast tumors does not depend on the type of p53 mutation and were similar for R172H mutation (17%) and R248Q p53 mutation (20%).

5) In contrast to breast cancer, the majority of R248Q sarcomas undergo spontaneous LOH indicating strong selective pressure for LOH in mutp53-driven tumors.

6) LOH in cancer cells coincides with stabilization of mutp53 protein suggesting that p53 LOH is the pre-requisite for mutp53 stabilization in tumors. LOH-mediated stabilization of mutp53 occurs independently of tissue of origin and/or the presence of additional oncogenes.

7) The enhanced LOH and its related mutant p53 protein stabilization in sarcomas of R248Q/+;ErbB2 mice correlates with shorter tumor onset and survival suggesting that p53 LOH is required for oncogenic mutp53 GOF activities.

8) In contrast to the notion of dominant-negative effect of mutp53 in heterozygosity, we found that presence of one wtp53 allele is sufficient to induce p53 target genes in mammary tumors. The expression of p53 target gene Mdm2 in R248Q/+;ErbB2 tumors could explain the low level of mutp53 in heterozygous tumors.

- What was the impact on the development of the principal discipline(s) of the project?

We completed the study on new R248Q;ErbB2 mouse model and published results in peer-reviewed journal Cell Death and Disease (impact factor 5,965). The second manuscript, describing mammary tumors cell lines, established during the implementation of proposed study, was submitted to Molecular Cancer Therapeutic (impact factor 5,764). After favorable review, the manuscript was revised and re-submitted.

- What was the impact on other disciplines?

Nothing to Report.

- What was the impact on technology transfer?

Nothing to Report.

- What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

As we described above, despite of our extensive expertise and numerous vigorous attempts we failed to establish continuous culture of MECs from H/+;ErbB2, -/+;ErbB2 and +/+;ErbB2 mice, likely due to transcriptional activity of wtp53 in heterozygosity. All wtp53 expressing MECs undergo senescence following

passage 3. Therefore, now we mainly will focus on cancer cell lines established from mammary tumors of H/+;ErbB2, -/+;ErbB2 and +/+; ErbB2 mice.

We encountered delay in hiring Postdoctoral Associate, which would have the appropriate experience and credentials. This was mainly due to the demanding selection criteria and protracted hiring process in Stony Brook Medical Center (job advertisement, interviewing of all potential candidates, negotiation and all necessary paperwork to complete hiring). As a result of very competitive selection, Amr Ghaleb was hired as a Research Scientist. Amr Ghaleb, a talented researcher with outstanding credentials and extensive lab experience will be valuable asset for the successful implementation of the proposed study.

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

- Significant changes in use or care of human subjects

Nothing to Report.

- Significant changes in use or care of vertebrate animals.

Nothing to Report.

- Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

Journal publications.

Alexandrova EM, Mirza SA, Xu S, Schulz-Heddergott R, **Marchenko ND**, Moll UM. p53 loss-of-heterozygosity is a necessary prerequisite for mutant p53 stabilization and gain-of-function in vivo. Cell Death Dis. 2017 Mar 9;8(3):e2661. doi: 10.1038/cddis.2017.80.

Alisha Yallowitz, Lucas Garcia, Evguenia M. Alexandrova, **Marchenko N.** Heat shock factor 1 confers resistance to lapatinib in ErbB2-positive breast cancer cells. Molecular Cancer Therapeutics. Under second revision.

Both publications contain acknowledgement of DOD support.

- Other Products

Novel R248Q; ErbB2 mouse model recapitulating human Her2 positive breast cancer was generated.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Name:	Natalia Marchenko
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12 months
Contribution to Project:	Dr. Marchenko was responsible for the overall administration, data analysis, coordination and direction of the project and lab work. Dr. Marchenko performed breeding and mouse colony maintenance, tumor specimens analysis, mammary epithelial cells isolation, manuscript preparation.
Funding Support:	DOD # BC151569
Name:	Euvgenia Alexandrova
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3 months
Contribution to Project:	As a collaborator Dr. Alexandrova was involved in generation, specimen tissue preparation and data analysis of R248Q;ErbB2 mice, manuscript preparation.
Funding Support:	NCI gran t# K22CA190653-01A1

Name:	Sulan Xu
Project Role:	Lab technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3 months
Contribution to Project:	Sulan was responsible for breeding and mouse colony maintenance, mouse genotyping, performed tissue embedding, cutting and IHC staining.
Funding Support:	
Name:	Lucas Garcia
Project Role:	Undergraduate Student, Stony Brook University
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	11 months
Contribution to Project:	Lucas Garcia performed Western blot analysis of cell lines, mice genotyping and assessment of p53 LOH status in cell lines.
Funding Support:	none
Name:	Julia Rosenfeld
Project Role:	Summer Undergraduate Student, Binghamton University State University of New York
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2 months
Contribution to Project:	Julia Rosenfeld performed Western blot analysis of cell lines.

Funding Support:	none
Name:	Ute Moll
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 month
Contribution to Project:	As a collaborator Dr. Moll participated in planning of experiments, discussions of data interpretations, manuscript preparation.
Funding Support:	NCI grant R01CA176647

- What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

AMR M. GHALEB, Ph.D.
CURRICULUM VITAE

Name: Amr M. Ghaleb

Address:

E-mail Address:

Citizenship:

TITLE:

2015-current Adjunct Assistant Professor, Suffolk County Community College, NY.
2011-2016 Research Assistant Professor, School of Medicine, Stony Brook University, NY.

PREVIOUS ACADEMIC APPOINTMENTS:

2008-2011 Research Associate, Division of Digestive Diseases, WBRB, Emory University School of Medicine, Atlanta, GA.
2003-2008 Post Doc fellow, Division of Digestive Diseases, WBRB, Emory University School of Medicine, Atlanta, GA. My training was in the role of Kruppel Like factors in carcinogenesis, inflammation, inflammation-induced tumorigenesis and obesity-related diseases.

EDUCATION:

1997-2003 **Ph. D.**, Cellular Biology Department, Biological Sciences Building, University of Georgia, Athens, GA.
Doctoral thesis title: **Chemoattraction between adult *Schistosoma mansoni* worms.**
1990-1995 **M.Sc.**, Biochemistry Department, Ain Shams University, Cairo, Egypt.
Thesis titled **Characterization of Murine Humoral Response to *Schistosoma hematobium* Antigens.** Research started and completed at U.S. Naval Medical Research Unit No.3 (NAMRU-3), Abbasseyya, Cairo, Egypt.
1985-1989 **B.Sc.**, Biochemistry Department, Ain Shams University, Cairo, Egypt.

RESEARCH INTEREST SYNOPSIS

My research work is focused on the role of the gastrointestinal epithelium in health and disease. I am currently studying the role of the zinc finger transcription factor Gut-enriched Kruppel-like Factor (GKLF or KLF4) in inflammatory bowel disease (IBD), digestive tract microbiome, inflammation-induced tumorigenesis and in the regulation of intestinal stem cells.

TEACHING EXPERIENCE:

2015-current Adjunct Assistant Professor at Suffolk County Community College. Teaching Human Anatomy and Physiology, level II.

- 2012-2016 Co-development and co-lecturing the “Laboratory Techniques in Cancer Biology” lecture series Bio-364.01 at Stony Brook University.
- 1997-2003 Teaching assistant at the Biology Dept, Biological Sciences Building, University of Georgia, Athens, GA 30602, USA.
 Courses taught: - Biology 1107 Lab for Biology major students
 - Human Anatomy and Physiology 2202 lab for Biology major and non-major students
 - Human Anatomy 2204 lab (cadaver based) for Biology major and non-major students
 - Vertebrate Comparative & Developmental Anatomy 3000 lab for Biology major students.
 - Biology 1103 Lab for Biology non-major students.
- 2001 Taught biology at the Medical College Admission Test (MCAT) level for the Princeton Review, 550 Pharr St. 120, Atlanta GA 30605, USA.
- 2000 Tutored Biology for the University Tutorial Services, Milledge Hall, UGA, Athens, GA 30602, USA.
- 1994-1995 Teaching assistant at the Biology Dept., The American University at Cairo, El-Tahreer Square, Cairo, Egypt.
- Additional courses from work experience include: parasitology, cell and molecular biology (including cancer biology, molecular techniques and proteomics) and vertebrate development.

WORK EXPERIENCE:

- 1995-1997 **Scientific Liaison & Field Application Scientist**, at Clinilab Company for Import and Export of Research, Clinical and Diagnostic Kits and Equipments, Maady, Cairo, Egypt.
- 1994-1995 **Clinical Laboratory Technologist**, at Dr. Moammena Kammel Clinical Lab, Mohandessein, Cairo, Egypt.
- 1991-1993 **Technical Assistant**, at Dr. Beverly Mangold lab, Basic Sciences Division, Schistosomiasis Branch, NAMRU-3, Abbasseyya, Cairo, Egypt.
Research interests: Identification and characterization of vaccine candidate antigens of *S. mansoni* and *S. hematobium*.
- 1992-1993 **Senior Laboratory Technologist**, Basic Sciences Division, Schistosomiasis Branch, NAMRU-3, Abbasseyya, Cairo, Egypt.
- 1990-1992 **Laboratory Technician**, in life cycle maintenance laboratory of Schistosomiasis Division, NAMRU-3, Abbasseyya, Cairo, Egypt.
- 1990 **Laboratory Animal Technician**, NAMRU-3, Abbasseyya, Cairo, Egypt.
- 1989-1990 **Clinical Laboratory Technologist**, at the clinical laboratory at the Abbasseyya Fever Hospital, Cairo, Egypt.
- 1987 **Summer Student**, at the Parasitology Division, NAMRU-3, Abbasseyya, Cairo, Egypt.

PUBLICATIONS:

1. **Ghaleb AM**, Elkarim EA, Bialkowska AB, Yang VW. 2016. KLF4 Suppresses Tumor Formation in Genetic and Pharmacological Mouse Models of Colonic Tumorigenesis. Molecular cancer research. 14(4):385-96.

2. Bialkowska AB, **Ghaleb AM**, Nandan MO, **Yang VW**. 2016. Improved Swiss-rolling Technique for Intestinal Tissue Preparation for Immunohistochemical and Immunofluorescent Analyses. *J Vis Exp*. Jul 13;(113).
3. García-Barros M, Coant N, Kawamori T, Wada M, Snider AJ, Truman JP, Wu BX, Furuya H, Clarke CJ, Bialkowska AB, **Ghaleb A**, Yang VW, Obeid LM, Hannun YA. 2016. Role of neutral ceramidase in colon cancer. *FASEB J*. Dec 30(12):4159.
4. Jes G. Kuruvilla, Chang-Kyung Kim, **Amr M. Ghaleb**, Agnieszka B. Bialkowska, Calvin J. Kuo, and Vincent W. Yang. 2016. Krüppel-like Factor 4 Modulates Development of BMI1+ Intestinal Stem Cell-Derived Lineage Following γ Radiation-Induced Gut Injury in Mice. *Stem Cell Repts*. 6(6):815-24.
5. Snider AJ, Bialkowska AB, **Ghaleb AM**, Yang VW, Obeid LM, Hannun YA. 2016. Murine Model for Colitis-Associated Cancer of the Colon. *Methods Mol Biol*. 1438:245-54.
6. Kuruvilla JG, **Ghaleb AM**, Bialkowska AB, Nandan MO, Yang VW. 2015. Role of Krüppel-like factor 5 in the maintenance of the stem cell niche in the intestinal crypt. *Stem Cell Transl Investig*. 2(2). pii: e839.
7. Mallipattu SK, Horne SJ, D'Agati V, Narla G, Liu R, Frohman MA, Dickman K, Chen EY, Ma'ayan A, Bialkowska AB, **Ghaleb AM**, Nandan MO, Jain MK, Daehn I, Chuang PY, Yang VW, He JC. 2015. Krüppel-like factor 6 regulates mitochondrial function in the kidney. *J Clin Invest*. 125(3):1347.
8. **Amr M. Ghaleb**, Agnieszka B. Bialkowska, Ashley J. Snider, Dmitri V. Gnatenko, Yusuf A. Hannun, Vincent W. Yang, Valentina A. Schmidt. 2015. IQ motif-containing GTPase-activating protein 2 (IQGAP2) is a novel regulator of colonic inflammation in mice. *Plos One*. 10(6):e0129314.
9. Daniel Talmasov, Zhang Xinjun, Bing Yu, Mandayam O. Nandan, Agnieszka B. Bialkowska, Enas Elkarim, Jes Kuruvilla, Vincent W. Yang, and **Amr M. Ghaleb**. 2015. Krüppel-like factor 4 is a radioprotective factor for the intestine following γ -radiation-induced gut injury in mice. *Am. J. Physiol. Gastrointestinal and Liver Physiology*. 308(2):121.
10. Nandan MO, **Ghaleb AM**, Bialkowska AB, Yang VW. 2015. Krüppel-like factor 5 is essential for proliferation and survival of mouse intestinal epithelial stem cells. *Stem Cell Res*. 14(1):10.
11. Snider AJ, Ali WH, Sticca JA, Coant N, **Ghaleb AM**, Kawamori T, Yang VW, Hannun YA, Obeid LM. 2014. Distinct roles for hematopoietic and extra-hematopoietic sphingosine kinase-1 in inflammatory bowel disease. *PLoS One*. 9(12):e113998.
12. **Amr M. Ghaleb**, Hamed Laroui, Didier Merlin and Vincent W. Yang. 2014. Genetic deletion of Klf4 in the mouse intestinal epithelium ameliorates dextran sodium sulfate-induced colitis and suppresses NF- κ B pathway inflammatory response. *Inflammatory Bowel Disease* 20(5):811.
13. Mandayam O. Nandan, **Amr M. Ghaleb**, Yang Liu, Agnieszka B. Bialkowska, Beth B. McConnell, Kenneth R. Shroyer, Sylvie Robine, Vincent W. Yang. 2014. Inducible intestine-specific deletion of Krüppel-like factor 5 is characterized by a regenerative response in adult mouse colon. *Developmental Biology* 387:191.
14. Enas A. El-Karim, Engda G. Hagos, **Amr M. Ghaleb**, Bing Yu, and Vincent W. Yang. 2013. Krüppel-like factor 4 regulates genetic stability in mouse embryonic fibroblasts. *Molecular Cancer* 12:89.

15. Shiwang Li, **Amr M. Ghaleb**, Jing He, Usha Bughami, Agnieszka B. Bialkowska, Vincent Yang and Harish C. Joshi. 2012. Chemoprevention of Familial Adenomatous Polyposis by EM011 in the *Apc^{Min/+}* Mouse Model. *Int. J. Cancer* 131(6):1435.
16. Pallavi Garg, Sabrina Jeppsson, Guillaume Dalmasso, **Amr Ghaleb**, Beth B McConnell, Vincent W Yang, Andrew T Gewirtz, Didier Merlin and Shanti V Sitaraman. 2011. MMP-9 modulates murine colitis associated cancer through Notch-1. *Gastroenterology* 141(4):1381.
17. McConnell, Beth B., Kim, Samuel S., **Ghaleb, Amr M.**, Nusrat, Asma, Nagai, Ryoza and Yang, Vincent W. 2011. Krüppel-like Factor 5 is a Key Factor for Maintenance of Crypt Architecture and Barrier Function in the Intestinal Epithelium. *Gastroenterology* 141(4):1302.
18. **Amr M. Ghaleb**, Beth B. McConnell, Klaus H. Kaestner, and Vincent W. Yang. 2011. Altered Intestinal Epithelial Homeostasis in Mice with Intestine-Specific Deletion of the Krüppel-Like Factor 4 Gene. *Developmental Biology* 349(2):310.
19. Engda G. Hagos, **Amr M. Ghaleb**, Amrita Kumar, Andrew S. Neish, and Vincent W. Yang. 2011. Expression profiling and pathway analysis of Krüppel-Like Factor 4 in mouse embryonic fibroblasts. *Am J Cancer Res.* 1(1):85.
20. Patel N, **Ghaleb AM**, Nandan MO, Yang VW. 2010. Expression of the Tumor Suppressor Kruppel-Like Factor 4 as a Prognostic Predictor for Colon Cancer. *Cancer Epidemiol Biomarkers Prev.* 19(10):2631.
21. Mandayam O Nandan, **Amr M Ghaleb**, Beth B McConnell, Nilesh V Patel, Sylvie Robine and Vincent W Yang. 2010. Kruppel-Like Factor 5 is a Crucial Mediator of Intestinal Tumorigenesis in Mice Harboring Combined *Apc^{Min}* and KRASV12 Mutations. *Molecular Cancer* 9:63.
22. E G Hagos, **A M Ghaleb**, W B Dalton, A B Bialkowska and V W Yang. 2009. Mouse embryonic fibroblasts null for the Krüppel-like factor 4 gene are genetically unstable. *Oncogene*, 28(9):1197.
23. Beth B. McConnell, Agnieszka B. Bialkowska, Mandayam O. Nandan, **Amr M. Ghaleb**, Frank J. Gordon and Vincent W. Yang. 2009. Haploinsufficiency of Krüppel-Like Factor 5 Rescues the Tumor-Initiating Effect of the *Apc^{Min}* Mutation in the Intestine. *Cancer Res.* 69:4125.
24. Songbai Lin, Dongsheng Wang, Smita Iyer, **Amr M. Ghaleb**, Hyunsuk Shim, Vincent W. Yang, Jerold Chun, and C. Chris Yun. 2009. LPA₂ promotes tumor formation in an experimental model of colitis-associated colon cancer. *Gastroenterology*, 136(5):1711.
25. **Ghaleb AM**, Yang VW. 2008. KLF4 (Kruppel-like factor 4 (gut)). *Atlas Genet Cytogenet Oncol Haematol*. URL : <http://AtlasGeneticsOncology.org/Genes/KLF4ID44316ch9q31.html>
26. **Amr M. Ghaleb**, Gaurav Aggarwal, Agnieszka B. Bialkowska, Mandayam O. Nandan, Vincent W. Yang. 2008. Notch Inhibits Expression of the Krüppel-Like Factor 4 Tumor Suppressor in the Intestinal Epithelium. *Molec. Cancer Res.* 6(12):1920.
27. **Ghaleb AM**, Yang VW. 2008. The Pathobiology of Krüppel-like Factors in Colorectal Cancer. *Curr. Colorectal Cancer Rep.* 4(2):59.
28. M. Nandan, B. McConnell, **A. Ghaleb**, A. Bialkowska, H. Sheng, J. Shao, B. Babbins, S. Robine, V. Yang. 2008. Krüppel-Like Factor 5 Mediates Cellular Transformation During Oncogenic KRAS-Induced Intestinal Tumorigenesis. *Gastroenterology*, 134(1):120.
29. **Amr M. Ghaleb**, Beth B. McConnell, Mandayam O. Nandan, Jonathan P. Katz, Klaus H. Kaestner, and Vincent W. Yang. 2007. Haploinsufficiency of Krüppel-Like Factor 4 Promotes Adenomatous Polyposis Coli-Dependent Intestinal Tumorigenesis. *Cancer Res.* 67(15):7147.

30. Rusovici R, **Ghaleb A**, Shim H, Yang VW, Yun CC. 2007. Lysophosphatidic acid prevents apoptosis of Caco-2 colon cancer cells via activation of mitogen-activated protein kinase and phosphorylation of Bad. *Biochim Biophys Acta*. 1770(8):1194.
31. McConnell BB, **Ghaleb AM**, Nandan MO, Yang VW. 2007. The diverse functions of Kruppel-like factors 4 and 5 in epithelial biology and pathobiology. *Bioessays*. 29(6):549.
32. Aneja R, **Ghaleb AM**, Zhou J, Yang VW, Joshi HC. 2007. p53 and p21 determine the sensitivity of nscapine-induced apoptosis in colon cancer cells. *Cancer Res*. 67(8):3862.
33. **Ghaleb AM**, Katz JP, Kaestner KH, Du JX, Yang VW. 2007. Kruppel-like factor 4 exhibits antiapoptotic activity following gamma-radiation-induced DNA damage. *Oncogene*. 26(16):2365.
34. Erika M. Whitney, **Amr M. Ghaleb**, Xinming Chen, and Vincent W. Yang. 2006. Transcriptional profiling of the cell cycle checkpoint gene Kruppel-Like factor 4 reveals a global inhibitory function in macromolecular biosynthesis. *Gene Expression*. 13:85.
35. Aneja R, Lopus M, Zhou J, Vangapandu SN, **Ghaleb A**, Yao J, Nettles JH, Zhou B, Gupta M, Panda D, Chandra R, Joshi HC. 2006. Rational design of the microtubule-targeting anti-breast cancer drug EM015. *Cancer Research*. 66(7):3782.
36. **Amr M. Ghaleb**, James Atwood III, Jorge Morales-Montor, and Raymond T. Damian. 2006. A 3kDa peptide is involved in the chemoattraction in vitro of the male *Schistosoma mansoni* to the female. *Microbes Infect*. 8 (9-10):2367.
37. Baig S, Damian RT, Morales-Montor J, **Ghaleb A**, Baghdadi A, White AC Jr. 2006. Protection from murine cysticercosis by immunization with a parasite cysteine protease. *Microbes Infect*. 8(12-13):2733.
38. Salman Baig, Raymond T. Damian, Jorge Morales-Montor, Rupal Mohandas, **Amr M. Ghaleb**, Meredith Welch, Jamil Talhouk, and A. Clinton White, Jr. 2006. Cysteine Proteinase Inhibitors in Murine Cysticercosis. *Antimicrobial Agents and Chemotherapy*. 50(8):2886.
39. **Ghaleb AM**, Nandan MO, Chanchevalap S, Dalton WB, Hisamuddin IM, Yang VW. 2005. Kruppel-like factors 4 and 5: the yin and yang regulators of cellular proliferation. *Cell Research*. 15(2):92.
40. Yoon HS, **Ghaleb AM**, Nandan MO, Hisamuddin IM, Dalton WB, Yang VW. 2005. Kruppel-like factor 4 prevents centrosome amplification following gamma-irradiation-induced DNA damage. *Oncogene*. 24(25):4017.
41. Morales-Montor J, Mohamed F, **Ghaleb AM**, Baig S, Hallal-Calleros C, Damian RT., 2001. In vitro effects of hypothalamic-pituitary-adrenal (HPA) axis hormones on *Schistosoma mansoni*. *J. Parasitology*. 87(5):1132.

INVITATIONS TO NATIONAL OR INTERNATIONAL CONFERENCES OR TO ACADEMIC INSTITUTIONS:

1. Invited lecturer at Colgate University, NY, October 2015.
2. FASEB Summer Research Conference, Snowmass, CO. August 2014. Critical roles of Kruppel-like factor 4 in the intestinal epithelium.

ABSTRACTS:

1. **Amr M. Ghaleb**, Enas A. Elkarim, Agnieszka B. Bialkowska, and Vincent W. Yang. Kruppel-like factor 4 suppresses intestinal tumorigenesis by regulating mTOR signaling

- pathway and DNA repair mechanisms. May 2016. The Annual Digestive Diseases Week, San Diego.
2. Jes Kuruvilla, Agnieszka B. Bialkowska, **Amr M. Ghaleb** and Vincent W. Yang. Krüppel-like factor 4 modulates the proliferation and survival of Bmi1+ stem cell derived crypts in the intestine following γ -radiation-induced gut injury in mice. May 2015. The Annual Digestive Diseases Week, D.C.
 3. Enas A. El-Karim, **Amr M. Ghaleb**, and Vincent W. Yang. Krüppel-like factor 4 (KLF4) regulates centrosome duplication by localizing to the centrosomes and is required for p53 centrosome localization. May 2015. The Annual Digestive Diseases Week, D.C.
 4. Daniel Talmasov, Xinjun Zhang, Bing Yu, Mandayam O. Nandan, Agnieszka Bialkowska, Enas Elkarim, Jes Kuruvilla, Vincent Yang, and **Amr Ghaleb**. Intestinal Krüppel-like factor 4 reduces cell death in the gut and contributes to survival following whole-body irradiation in mice. August 2014. FASEB Summer Research Conference, Snowmass, CO.
 5. Mandayam O. Nandan, **Amr M. Ghaleb**, Agnieszka B. Bialkowska and Vincent W. Yang. Krüppel-like factor 5 deletion in mouse intestinal stem cells results in short-term loss of proliferation and long-term depletion of stem cells. August 2014. FASEB Summer Research Conference, Snowmass, CO.
 6. **Amr M. Ghaleb** and Vincent W. Yang. Intestine-specific deletion of Klf4 renders mice susceptible to inflammation-associated tumorigenesis and increases intestinal adenoma multiplicity and colonic adenoma incidence in *Apc^{Min/+}* mice. May 2014. The Annual Digestive Diseases Week, Chicago, IL.
 7. Mandayam O. Nandan, **Amr Ghaleb**, Agnieszka Bialkowska, Vincent W. Yang. Intestinal Stem Cell (LGR5)-Specific Deletion of Krüppel-Like Factor 5 in Mice Results in Acute Loss of Proliferation and Chronic Depletion of Stem Cells. May 2014. The Annual Digestive Diseases Week, Chicago, IL.
 8. **Amr M. Ghaleb**, Hamed Laroui, Poonam Rakhya, Didier Merlin, Vincent W. Yang. Amelioration of dextran sodium sulfate-induced colitis in mice by genetic deletion of Klf4 in the intestinal epithelium or by oral delivery of nanoparticles loaded with Klf4–siRNA to the colonic epithelial cells. May 2013. The Annual Digestive Diseases Week, Orlando, FL.
 9. Enas A. El-Karim, **Amr M. Ghaleb**, Engda G. Hagos, and Vincent W. Yang. Krüppel-Like Factor 4 is necessary and sufficient for re-establishing genetic stability in mouse embryonic fibroblasts null for Klf4. May 2013. The Annual Digestive Diseases Week, Orlando, FL.
 10. Daniel Talmasov, **Amr M. Ghaleb**, Bing Yu, Mandayam O. Nandan, Vincent W. Yang. Krüppel-like factor 4 is a radio-protective factor of the intestine following γ radiation-induced gut injury in mice. May 2013. The Annual Digestive Diseases Week, Orlando, FL.
 11. **Amr M. Ghaleb**, Hamed Laroui, Didier Merlin, Vincent W. Yang: Deletion of the Krüppel-like factor 4 gene in colonic epithelium protects mice from dextran sulfate sodium (DSS)-induced colitis. August 2012. FASEB Summer Research Conference, Snowmass, CO.
 12. **Amr M. Ghaleb**, Hamed Laroui, Didier Merlin, Vincent W. Yang, Hamed Laroui, Didier Merlin, Vincent W. Yang: Deletion of the Krüppel-like factor 4 gene in colonic epithelium protects mice from dextran sulfate sodium (DSS)-induced colitis. May 2012. The Annual Digestive Diseases Week, San Diego, CA.
 13. **Amr M. Ghaleb**, Beth B. McConnell, and Vincent W. Yang: Intestine-specific deletion of the Krüppel-like factor 4 (Klf4) gene Protects Mice from Dextran Sulfate Sodium (DSS)-Induced Colitis. May 2011. The Annual Digestive Diseases Week, Chicago, IL.
 14. Mandayam O. Nandan, **Amr M. Ghaleb**, Vincent W. Yang: Mice with intestine-specific expression of K-Ras^{G12D} from the endogenous promoter exhibit a hyperproliferative

- intestinal phenotype and develop spontaneous intestinal polyposis. May 2011. The Annual Digestive Diseases Week, Chicago, IL.
15. **Amr M. Ghaleb**, Beth B. McConnell, Jonathan P. Katz, Klaus H. Kaestner and Vincent W. Yang: Intestine-Specific Deletion of the Mouse Krüppel-Like Factor 4 Gene Results in Altered Homeostasis of Intestinal Epithelial Cells. May 2010. The Annual Digestive Diseases Week, New Orleans, LA.
 16. Nandan, Mandayam O.; **Ghaleb, Amr**; McConnell, Beth B.; Yang, Vincent W: Krüppel-Like Factor Is a Crucial Mediator of Intestinal Tumorigenesis in Mice Harboring Combined APC and KRAS Mutations. May 2010. The Annual Digestive Diseases Week, New Orleans, LA.
 17. **Amr Ghaleb**, Beth B. McConnell, Jonathan P. Katz, Klaus H. Kaestner, Vincent W. Yang: The Krüppel-like factor 4 and its role in intestinal homeostasis. August 2010. FASEB Summer Research Conference, Steamboat Springs, CO.
 18. **Ghaleb, Amr M.**; Aggarwal, Gaurav; Bialkowska, Agnieszka; Nandan, Mandayam O. and Yang, Vincent W: Notch signaling regulates expression of the Krüppel-like factor 4 gene in the intestinal epithelium. May 2008. The Annual Digestive Diseases Week, San Diego, CA.
 19. McConnell, Beth B.; Bialkowska, Agnieszka; Nandan, Mandayam O.; **Ghaleb, Amr M.**; and Yang, Vincent W: Haploinsufficiency of Krüppel-like factor 5 Inhibits Adenoma Formation in *Apc^{Min/+}* Mice by Modulating β -Catenin Activity. May 2008. The Annual Digestive Diseases Week, San Diego, CA.
 20. Hagos, Engda G., **Ghaleb, Amr M.**, Dalton, W. Brian, Katz, Jonathan P., Kaestner, Klaus H., and Yang, Vincent W: Mouse Embryonic Fibroblasts Null for the Krüppel-Like Factor 4 Alleles are Genetically Unstable. May 2008. The Annual Digestive Diseases Week, San Diego, CA.
 21. **Ghaleb, Amr M.**; McConnell, Beth B.; Nandan, Mandayam O.; Katz, Jonathan P.; Kaestner, Klaus; Yang, Vincent W: Role of Krüppel-Like Factor 4 in Wnt and Notch signaling in the intestinal tract. May 2007. The Annual Digestive Diseases Week, Washington D.C.
 22. Nandan, Mandayam O.; Bialkowska, Agnieszka; Babbin, Brian A.; McConnell, Beth B.; **Ghaleb, Amr M.**; Yang, Vincent W: Increased Expression of Krüppel-Like Factor 5 in Human Colon Cell Lines with K-Ras Mutation and in Primary Human Colorectal Cancer. May 2007. The Annual Digestive Diseases Week, Washington D.C.
 23. **Amr M. Ghaleb** and Vincent W. Yang: Kruppel-like factor 4 binds and suppresses Bax expression following gamma irradiation-induced DNA damage. May 2006. The Annual Digestive Diseases Week, Los Angeles, CA.
 24. **A.M. Ghaleb**, V.W. Yang: Suppression of apoptosis by Krüppel-like factor 4 following gamma irradiation-induced DNA damage. May 2005. The Annual Digestive Diseases Week, Chicago, IL.
 25. Hong S. Yoon, **Amr M. Ghaleb**, Mandayam O. Nandan, Irfan M. Hisamuddin and Vincent W. Yang: Kruppel-like factor 4 prevents centrosome amplification following γ irradiation-induced DNA damage. May 2005. The Annual Digestive Diseases Week, Chicago, IL.
 26. **Amr M. Ghaleb** and Raymond T. Damian: Dynamics of the chemoattraction between male and female adult *Schistosoma mansoni* worms in vitro. October 2000. The Annual Immunoparasitology Workshop, Fancy Gap, VA.
 27. **Amr M. Ghaleb** and Raymond T. Damian: Dynamics of the chemoattraction between male and female adult *Schistosoma mansoni* worms in vitro. May 2000. The 10th Annual Molecular Parasitology/Vector Biology Symposium, Athens, GA.

28. **Amr M. Ghaleb** and Raymond T. Damian: Chemoattraction between male and female adult *Schistosoma mansoni* worms. October 1999. The Annual Immunoparasitology Workshop, Fancy Gap, VA.

AWARDS:

- 2016 Early Stage Investigator Certificate of Recognition for Scientific Accomplishment at the Annual Digestive Diseases Week, San Diego, CA.
 2012 Poster of Distinction presentation award at the Annual Digestive Diseases Week, San Diego, CA.
 2010 Outstanding Achievement merit award from Emory University.
 2008 The American Gastroenterological Association (AGA) travel award.

MENTORING

- 2013-2016 Jes Kuravala, Junior Postdoctoral fellow at Stony Brook University
 2012-2013 Xinjun Zhang, Visiting Scholar at Stony Brook University.
 2012-2013 Daniel Talmasov, Medical student, and Syed Karim, Senior graduate student at Stony Brook University.
 2007-2010 Engda Hagos, Junior Postdoctoral Fellow at Emory University.
 2007 Gaurav Aggarwal, Junior Gastroenterology Fellow at Emory University.
 2006 Candice Elam, Graduate student at Emory University.

AD-HOC REVIEWER FOR SCIENTIFIC JOURNALS

Gastroenterology, Nature Medicine, American Journal of Physiology, PLOS, Hepatology, British Journal of Cancer, Scientific Reports.

PROFESSIONAL AFFILIATIONS/MEMBERSHIPS:

- 2006-2010 The American Gastroenterological Association (AGA).

RESEARCH SUPPORT

Pending:

- | | | |
|---|--------------------|-----------------------|
| 2016 | | R01 DK113138-01 |
| Amr Ghaleb (PI) | Role: PI | |
| Molecular Mechanisms of IBD and Intestinal Epithelium Repair. | | |
| 2016 | | Department of Defense |
| Valentina Schmidt (PI) | Role: Collaborator | |
| Role of IQGAP2 in Colonic Inflammation. | | |

Completed Research Support

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|---|---------------|------------------------|
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p53 loss-of-heterozygosity is a necessary prerequisite for mutant p53 stabilization and gain-of-function *in vivo*

Evguenia M Alexandrova¹, Safia A Mirza¹, Sulan Xu¹, Ramona Schulz-Heddergott², Natalia D Marchenko¹ and Ute M Moll^{*,1,2}

Missense mutations in *TP53* comprise > 75% of all p53 alterations in cancer, resulting in highly stabilized mutant p53 proteins that not only lose their tumor-suppressor activity, but often acquire oncogenic gain-of-functions (GOFs). GOF manifests itself in accelerated tumor onset, increased metastasis, increased drug resistance and shortened survival in patients and mice. A known prerequisite for GOF is mutant p53 protein stabilization, which itself is linked to aberrant protein conformation. However, additional determinants for mutant p53 stabilization likely exist. Here we show that in initially heterozygous mouse tumors carrying the hotspot GOF allele R248Q (p53Q/+), another necessary prerequisite for mutant p53 stabilization and GOF *in vivo* is loss of the remaining wild-type p53 allele, termed loss-of-heterozygosity (LOH). Thus, in mouse tumors with high frequency of p53 LOH (osteosarcomas and fibrosarcomas), we find that mutant p53 protein is stabilized (16/17 cases, 94%) and tumor onset is significantly accelerated compared with p53+/- tumors (GOF). In contrast, in mouse tumors with low frequency of p53 LOH (MMTV-Neu breast carcinomas), mutant p53 protein is not stabilized (16/20 cases, 80%) and GOF is not observed. Of note, human genomic databases (TCGA, METABRIC etc.) show a high degree of p53 LOH in all examined tumor types that carry missense p53 mutations, including sarcomas and breast carcinomas (with and without HER2 amplification). These data – while cautioning that not all genetic mouse models faithfully represent the human situation – demonstrate for the first time that p53 LOH is a critical prerequisite for missense mutant p53 stabilization and GOF *in vivo*.

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Missense mutations in *TP53* (mutp53) comprise > 75% of all p53 alterations in cancer, resulting in highly stabilized mutant p53 proteins that not only lose their tumor-suppressor activity, but often acquire oncogenic gain-of-functions (GOFs).^{1–5} GOF activities promote cancer metabolism, stemness, and malignant progression and invasion. This results in accelerated tumor onset, increased metastasis, increased drug resistance and shortened survival in patients and mice.^{5–7} Accordingly, mutp53 knockin mice carrying the human hotspot missense R248Q mutation have significantly earlier tumor onset and shorter survival than p53-null mice.⁵ In agreement, in human patients with sporadic cancers across six major tumor entities, cancers with GOF mutp53 R282 and R248 alleles show a twofold higher hazard ratio (i.e., increased mortality) compared with cancers with loss-of-function (LOF) mutp53 alleles (nonsense, frameshift and deletion mutations).⁸ Similarly, germline Li–Fraumeni syndrome (LFS) patients carrying R248Q mutp53 exhibit markedly faster tumor onset by 10.5 years and higher tumor numbers per person than LFS patients carrying LOF mutp53.⁵

Conversely, mutp53 elimination significantly suppresses tumor growth and metastasis and markedly extends survival in various mouse models.^{7,9,10} For example, mutp53 depletion by RNAi has strong cytotoxic effects in human cancer cell lines *in vitro* and in xenografts.⁷ In allografts, knockdown of mutp53 in *Kras*^{G12D} pancreatic cancer cells strongly reduces their metastatic ability.⁹ Finally, in a conditional inactivatable ('floxed') autochthonous mouse model, ablation of the

R248Q knockin allele extends survival by 37%, induces regression or stagnation of advanced tumors and strongly suppresses metastasis.¹⁰

A known prerequisite for mutp53 GOF is its massive constitutive protein stabilization specifically in tumors – but not in normal cells – of knockin mice.^{6,11,12} Currently about 11 million patients worldwide live with cancers expressing highly stabilized mutp53, raising the question: what factors determine mutp53 stabilization leading to oncogenic GOF? One established determinant are the aberrant protein conformations of both the structural and DNA-contact classes of missense mutant p53 proteins, requiring constitutive chaperone complexing (with, e.g., Hsp90 and Hsp40) to protect them from their E3 ubiquitin ligases Mdm2 and CHIP and proteasomal degradation.^{10,13–18} Indeed, pharmacological inhibition of the HSP90 chaperone machinery destabilizes mutp53, leading to 48% and 59% prolonged survival in R175H and R248Q knockin mice, respectively.¹⁰ We hypothesized that besides aberrant conformation additional determinants of mutp53 stabilization likely exist. Here we show that loss of the remaining wild-type p53 (wtp53) allele, termed loss-of-heterozygosity (LOH), is also critical for missense mutp53 stabilization and GOF *in vivo*.

Results

TCGA, METABRIC and other databases of sporadic human cancer show wtp53 allele loss (LOH) in the majority of

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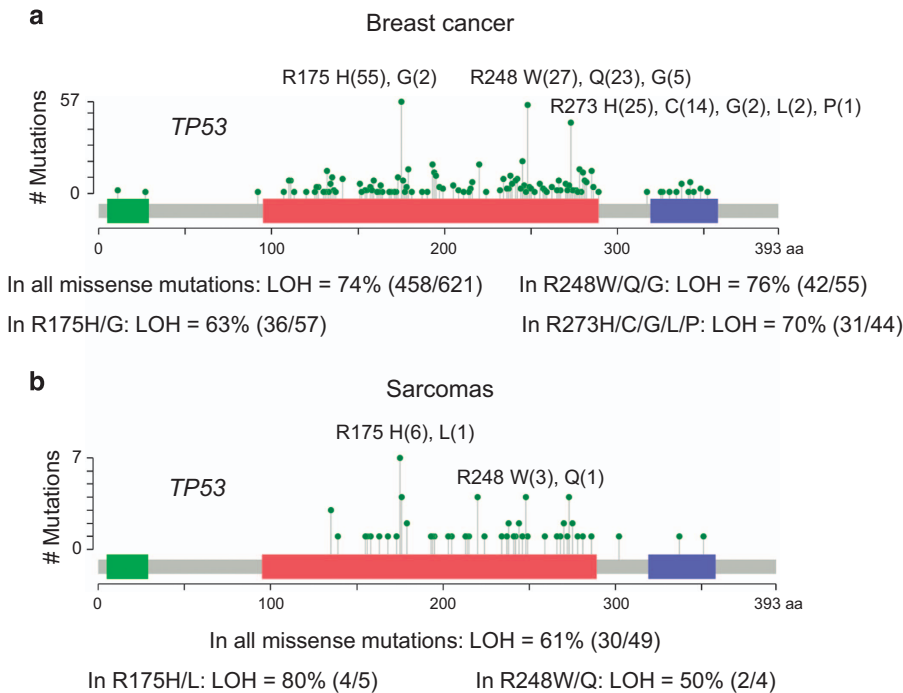


Figure 1 Analysis of the databases of sporadic human breast cancer (a) and sarcomas (b) (METABRIC, provisional TCGA and others, see Materials and Methods section) show a high frequency of wtp53 allele loss (LOH) in missense mutp53 tumors

Table 1 Frequency of p53 LOH in human HER2-positive breast cancer carrying concomitant missense mutp53

Database	Cases with p53 LOH	Total number of cases	LOH frequency
METABRIC	97	124	78.2%
TCGA provisional	38	40	95.0%
Total	135	164	82.3%

Table 2 Frequency of p53 LOH in human high-grade serous ovarian adenocarcinoma carrying concomitant missense mutp53

Database	Cases with p53 LOH	Total number of cases	LOH frequency
TCGA provisional	206	274	75.2%

missense mutp53 tumors, including ovarian cancer, breast cancer and sarcomas (Figure 1, Tables 1 and 2). Specifically, in human HER2 breast cancer with concomitant missense mutp53, wtp53 LOH occurs in 82.3% of patients (Table 1). Thus, we hypothesized that LOH is a second determinant of mutp53 stabilization and GOF *in vivo*.

To test this, we combined the heterozygous hotspot GOF allele R248Q ('p53Q/+')^{5,10} with the MMTV-Neu ('Neu') oncogene¹⁹ expressing additional wild-type HER2 copies selectively in the mammary gland, as mutp53 has a strong prognostic value in HER2-positive breast cancer, that is, significantly increased mortality.²⁰ Although p53Q/+;Neu mice

developed breast cancer faster than p53+/+;Neu mice, surprisingly breast cancer latency between p53Q/+;Neu and p53 -/+;Neu siblings was similar (Figure 2b), suggesting that mutp53 R248Q did not exert a dominant-negative (DN) effect over wtp53 but simply behaved as a LOF allele in Neu-driven breast tumorigenesis *in vivo*, hence the curves overlap.

However, about half of p53Q/+;Neu and p53 -/+;Neu mice did not develop breast cancer but instead developed osteosarcomas and fibrosarcomas, which originate from mesenchymal tissues where MMTV-Neu is not expressed (Figure 2a). Notably, sarcoma onset was faster in p53Q/+;Neu compared with p53 -/+;Neu mice, indicating either a DN effect of mutp53 over wtp53 or, alternatively, wtp53 LOH resulting in mutp53 GOF specifically in sarcoma. Importantly, this survival difference between sarcoma and breast cancer correlated with mutp53 stabilization in nearly all examined sarcomas (94%, 16/17), but only in rare breast carcinomas (20%, 4/20), even within the same animal (Figure 3a, e.g., animal #1248).

Thus, we asked whether sarcomas are more prone to p53 LOH than breast tumors. Indeed, qPCR of genomic DNA showed that p53 LOH occurs in all sarcomas, but rarely in breast cancer (Figure 3b). Moreover, the few breast tumors that did stabilize mutp53 also underwent p53 LOH. Together, this strongly suggests that LOH is a critical prerequisite for mutp53 stabilization and GOF (Figure 3c). To corroborate our LOH data, we analyzed p53 target genes as another readout for the remaining wtp53 allele activity (Figure 4). Indeed, all tumors with stabilized mutp53, including the single 'outlier' breast cancer tested, had reduced or undetectable *Mdm2* and *p21* levels, respectively, and sarcomas also had reduced *Bax* and *Puma* expression correlating with their LOH.

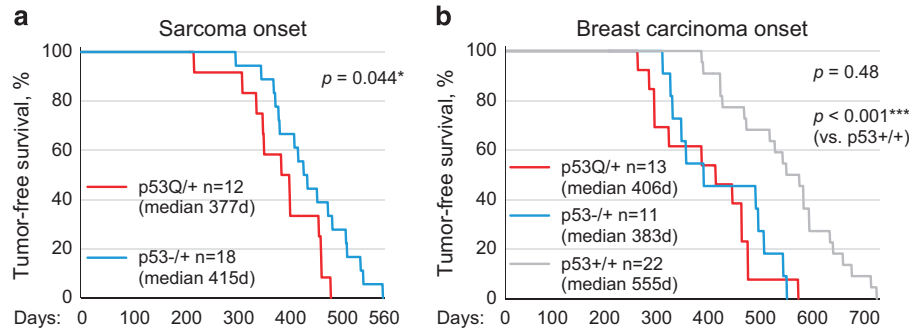


Figure 2 Survival curves analyzing tumor onset of sarcomas and breast carcinomas in p53Q/+;Neu, p53-/-;Neu and p53+/+;Neu mouse cohorts. (a) Sarcoma onset is faster in p53Q/+;Neu compared with p53-/-;Neu mice. This indicates either a DN effect over wtp53 or, alternatively, p53 LOH resulting in mutp53 GOF specifically in sarcoma. (b) Breast cancer latency in p53Q/+;Neu and p53-/-;Neu siblings is similar, reflecting that the majority of p53Q/+ breast tumors did not undergo LOH (see Figure 3b) in contrast to human breast cancer, and also did not exert a DN effect over wtp53 but simply behaved as a LOF allele. Kaplan-Meier analysis; n, number of mice; P, log rank statistics

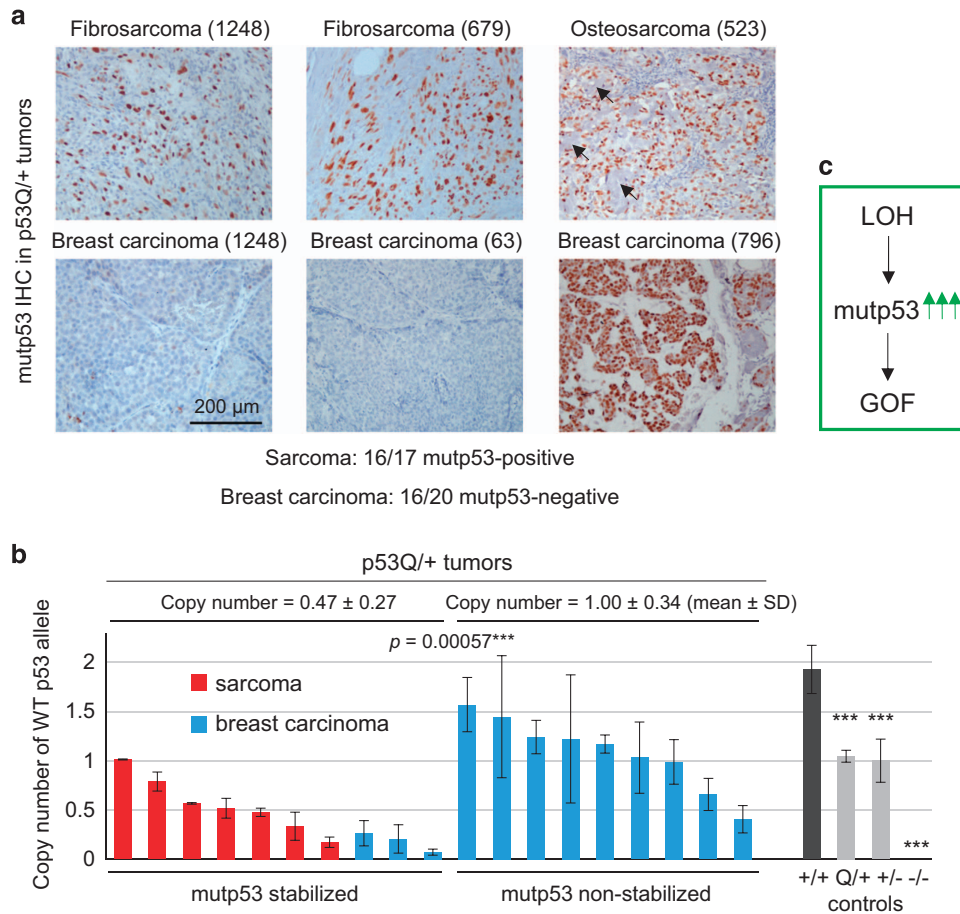


Figure 3 Loss of wtp53 allele is required for missense mutant p53 stabilization and GOF. (a) The vast majority of sarcomas (16/17 cases, 94%) have stabilized mutp53. In contrast, the majority of breast carcinomas (16/20 cases, 80%) do not. Immunohistochemistry for mutp53. Mouse identity in parentheses. Arrows indicate the osteoid in osteosarcoma. (b) Analysis of wtp53 copy number in sarcomas and breast carcinomas of p53Q/+;Neu mice by quantitative genotyping. Tumors with mutp53 stabilization (all sarcomas and three breast cancers tested) have significantly higher LOH than tumors without mutp53 stabilization (majority of breast cancers). Note, as sarcomas have high normal stroma contamination (top, blue mutp53-negative stromal cells, which do not have LOH), the actual LOH in sarcomas is most likely even higher because of dilution of the tumor genotype, causing LOH underestimation. For the same reason, copy numbers of the two highest sarcoma cases (two left red bars) are likely inflated. The wtp53 signal was normalized to the Rosa26 signal. Tail biopsies from p53+/+ (two wt alleles), p53Q/+, p53-/- (one wt allele) and p53-/- mice (no wt alleles) were used as normal control tissues without LOH. Bars represent mean \pm S.D. of two technical replicas of individual cases. $^{***}P < 0.001$. (c) Schematic diagram of the proposed mechanism for mutp53 stabilization and GOF in heterozygous tumors. Loss of the wtp53 allele (LOH) causes accumulation of highly stabilized mutp53 protein, which triggers tumor development and is the principle mechanism and prerequisite of GOF

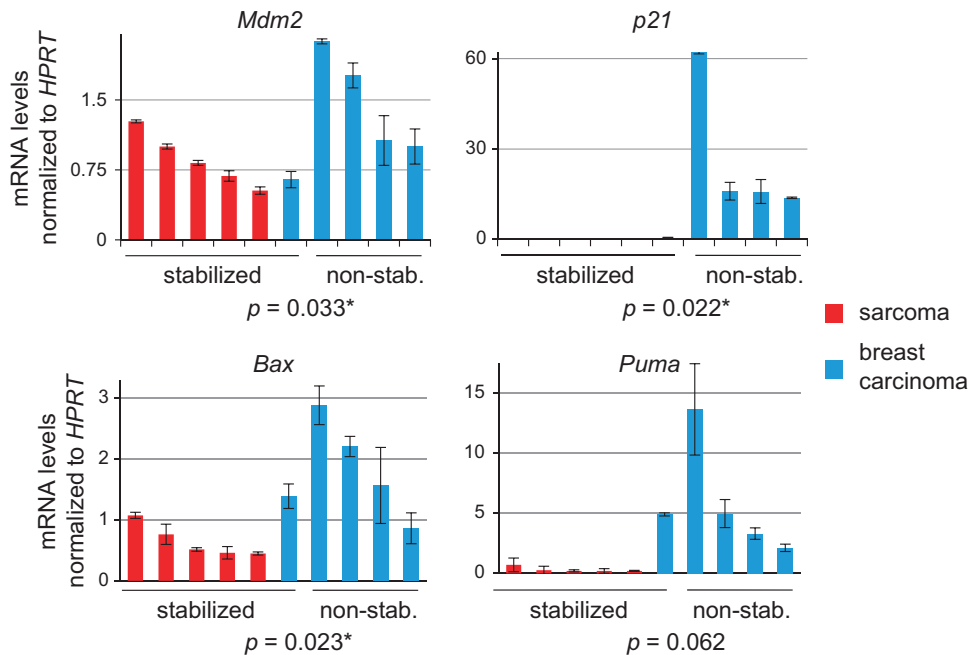


Figure 4 Real-time qPCR analysis of wtp53 target genes *Mdm2*, *p21*, *Bax* and *Puma* shows that their expression is largely decreased in samples with mutp53 stabilization compared with samples without mutp53 stabilization

Discussion

In sum, we propose that p53 LOH is a necessary prerequisite for mutp53 stabilization and GOF activity *in vivo* (Figure 3c). Indeed, we find that *TP53* LOH is a frequent event in human cancers with missense mutp53, including sarcomas (61%), breast cancer with or without HER2 amplification (up to 82%) and ovarian cancer (75%) (Figure 1, Tables 1 and 2). This high LOH frequency coincides with mutp53 protein stabilization^{21,22} and GOF in human cancers.^{5,8} Our *TP53* LOH data are in agreement with earlier reports finding 60% *TP53* LOH in LFS patients,²³ 81% in sporadic breast cancer patients (all molecular subtypes pooled)²⁰ and 93% across 10 sporadic human cancer types,²⁴ all expressing missense mutp53. Note that the latter study with the highest frequency includes ‘copy neutral’ *TP53* LOH (defined as reduced wtp53 mRNA expression but genomic copy present) and also corrects for dilutional effects from stromal contamination.²⁴ This suggests that conventional and even quantitative real-time PCR – which we used in our analysis – likely underestimate true functional p53 LOH.

In full agreement with the human data, sarcomas in our mouse model also exhibit GOF because they undergo LOH, which enables mutp53 stabilization. Similarly, Shetzer *et al.*²⁵ found that isolated mesenchymal stem cells from heterozygous R175H/+ mice form subcutaneous tumors only after they undergo wtp53 LOH. How mechanistically p53 LOH induces mutp53 stabilization awaits further investigation. A possible contributor could be reduced expression of the wtp53 target gene *Mdm2* (Figure 4), the main ubiquitin ligase for both wtp53 and mutp53.^{11,16}

Although a few murine breast cancer cases in our MMTV-Neu model (4/20) did undergo LOH and exhibited mutp53

stabilization, for unknown reasons the majority (16/20) lacked LOH and therefore lacked mutp53 stabilization. We speculate that the pressure for p53 LOH is eliminated because of the Neu oncogene. This gives us pause that not all mouse models faithfully mimic the human genetic constellation for every tissue type, as the MMTV-Neu model contrasts with human breast cancer, which exhibit prominent LOH despite the presence of other oncogenic drivers (Figure 1a, Table 1).²⁰

Materials and Methods

TP53 LOH analysis in sporadic human cancers. *TP53* LOH in sporadic human cancers was analyzed using the cBioPortal tool (www.cbioportal.org). The breast cancer data set included METABRIC and provisional TCGA databases, with 3014 samples with known mutant p53 status in total. The sarcoma data set included provisional TCGA, MSKCC/Broad Institute, Institut Curie and other databases, with 710 samples with known mutp53 status in total.

Animals. Hotspot knockin mice harboring human exons 4–9 and the p53 R248Q missense mutation (‘Q’ allele) and p53 –/– mice, both on pure C57Bl/6 background, were previously described.^{5,10} MMTV-Neu (‘Neu’) transgenic mice on pure FVB/N background were from Jackson Laboratories (Bar Harbor, ME, USA) (FVB/N-Tg(MMTVneu)202Mul/J).¹⁹ To obtain p53Q/+;Neu/+ and control p53+/–; Neu/+ mice, parental p53 R248Q/+ and p53 –/+ strains were first crossed to obtain p53 R248Q/– mice, followed by cross with Neu/Neu mice. Only female mice were used for all experiments. Animals were monitored weekly to determine their breast cancer and sarcoma onset and were promptly killed when their tumors reached 2 cm³ in volume or when animals appeared moribund. All animals were treated humanely and according to the guidelines issued by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University.

Immunohistochemistry and histology. For immunohistochemical analysis, freshly dissected tissues were formalin fixed, paraffin embedded and sectioned (5 μm). Slides were deparaffinized and boiled in citrate buffer (10 mM, pH 6.0, 35 min) for antigen retrieval, blocked in 10% goat serum and incubated with the primary antibody (mutp53, Santa Cruz, Dallas, TX, USA, FL393, sc-6243, dilution 1:500) for 2 h at room temperature. After PBS washing, slides were incubated with

biotinylated secondary antibody and HRP-streptavidin using the Histostain SP Broad Spectrum kit (Invitrogen, Carlsbad, CA, USA, 959943B), stained with DAB substrate with hematoxylin counterstain and coverslipped. In addition, cancer type (breast cancer versus osteosarcoma or fibrosarcoma) was determined by H&E staining (data not shown).

Quantitative LOH analysis. Genomic DNA was isolated from sarcomas, breast carcinomas and control tails using DNeasy Blood and Tissue kit (Qiagen, 69506) and quantified by spectrophotometer. Quantitative real-time PCR was performed in duplicates with QuantiTect SYBR Green Mix (Qiagen, Germantown, MD, USA, 204143) on the MJ Research DNA Engine Opticon 2 machine, using 8 ng genomic DNA and the following mouse wtp53 allele-specific primer pairs: 5'-ACACGCTGGTGGTACCTTAT-3' (forward) and 5'-TATACTCAGAGCCGGCCT-3' (reverse). These wtp53 primers anneal to mouse exons 5 and 6 and do not recognize the humanized mtp53 allele. For all samples, the wtp53 signal was normalized to the Rosa26 signal measured by the following primers: 5'-AAAGTCGCTCTGAGTTGTAT-3' (forward) and 5'-GGAGCGGGAGAAATGGA TATG-3' (reverse).

Statistical analysis. Kaplan–Meier analysis and log rank statistics was used to analyze tumor onset. Unpaired two-tailed Student's *t*-test was used to analyze p53 LOH and expression of p53 target genes. **P* < 0.05, ****P* < 0.001.

Conflict of Interest

The authors declare no conflict of interest.

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Heat shock factor 1 confers resistance to lapatinib in ErbB2-positive breast cancer cells

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ABSTRACT (246/250 words)

Despite success of Her2-targeted therapies such as lapatinib, resistance remains a major clinical concern. Multiple compensatory receptor tyrosine kinase (RTK) pathways are known to contribute to lapatinib resistance. The heterogeneity of these adaptive responses is a significant hurdle for finding most effective combinatorial treatments. The goal of this study was to identify a unifying molecular mechanism whose targeting could help prevent and/or overcome lapatinib resistance. Using the MMTV-ErbB2;mutant p53 (R175H) *in vivo* mouse model of Her2-positive breast cancer, together with mouse and human cell lines, we compared lapatinib-resistant vs. lapatinib-sensitive tumor cells biochemically and by kinome arrays and evaluated their viability in response to a variety of compounds affecting heat shock response.

We found that multiple adaptive RTKs are activated in lapatinib-resistant cells *in vivo*, some of which have been previously described (Axl, MET) and some were novel (PDGFR α , PDGFR β , VEGFR1, MUSK, NFGFR). Strikingly, all lapatinib-resistant cells show chronically activated HSF1 and its transcriptional targets, heat shock proteins (HSPs), and, as a result, superior tolerance to proteotoxic stress. Importantly, lapatinib-resistant tumors and cells retained sensitivity to Hsp90 and HSF1 inhibitors, both *in vitro* and *in vivo*, thus, providing a unifying and actionable therapeutic node. Indeed, HSF1 inhibition simultaneously downregulated ErbB2, adaptive RTKs and mutant p53, and its combination with lapatinib prevented development of lapatinib resistance *in vitro*. Thus, the kinome adaptation in lapatinib-resistant Her2-positive breast cancer cells is governed, at least in part, by HSF1-mediated heat shock pathway, providing a novel potential intervention strategy to combat resistance.

INTRODUCTION

Human epidermal growth factor receptor 2 (Her2, aka ErbB2 in mice) is overexpressed in about 25% of sporadic human breast cancer cases, which correlates with poor prognosis (1). Several Her2-targeted therapies are available in a clinic that are known to improve patients' outcomes, including a dual ErbB2/EGFR kinase inhibitor lapatinib (2). However, about third of patients have a primary resistance to lapatinib and the rest develops secondary resistance within a year on lapatinib treatment (2). Therefore, lapatinib resistance remains a major concern for its clinical utilization.

Multiple mechanisms of lapatinib resistance are described in the literature. They primarily involve compensatory activation of various receptor tyrosine kinases (RTKs), such as ErbB3, IGF1R, MET, FGFR2, FAK, Axl, as well as other mechanisms, e.g. microRNAs, cancer stem cells, cell cycle, tumor metabolism etc. (2). Importantly, not a single, but multiple tyrosine kinases have been shown to be activated in response to lapatinib (3). Also, the substantial heterogeneity among adaptive RTKs exists in different cell lines in response to lapatinib (3). This represents a major hurdle for the development of successful combinatorial strategies to reverse and/or prevent lapatinib resistance. Hence, identification and targeting of an upstream effector governing the kinome adaption in response to ErbB2 inhibition by lapatinib would help to overcome the clinical dilemma of heterogeneity in compensatory pathways.

Our previous studies identified heat shock factor 1 (HSF1) as a key effector of ErbB2 signaling (4-6). HSF1 is a transcription factor that controls a broad spectrum of pro-survival events essential for protecting cells from proteotoxic stress, which is caused by the accumulation of misfolded proteins in cancer cells. Specifically, HSF1 activates transcription of genes that regulate protein homeostasis, including the molecular chaperones heat shock proteins (HSPs) Hsp27, Hsp70 and Hsp90 (7). Beyond protein folding, HSF1 also drives broad cancer-specific pro-survival programs that support oncogenic processes such as cell cycle regulation, cell signaling pathways, metabolism, adhesion, and protein translation (8,9). The impact of HSF1 on ErbB2 driven mammary tumorigenesis was unequivocally proven by *in vivo* studies. The genetic ablation of HSF1 suppresses mammary hyperplasia and reduces tumorigenesis in ErbB2 transgenic mice *in vivo* (10). Consistently, the stability of ErbB2 protein is shown to be maintained by transcriptional targets of HSF1: Hsp70, Hsp90 (11) and Hsp27 (7).

Mutations in the *TP53* gene (mutp53) are the most frequent genetic events in sporadic Her2-positive breast cancer in humans (72% (12)). Interestingly, the frequency of Her2-positive breast cancer is significantly increased in Li-Fraumeni syndrome patients that carry germ-line *TP53* mutations compared to the general population (without germline mutp53) (13). Altogether, this points to cooperativity of ErbB2 and mutp53 in Her2-positive breast cancer. Indeed, *TP53* mutations correlate with poor patient outcomes due to a more aggressive disease and increased susceptibility to metastasis (14).

To faithfully recapitulate human Her2-positive breast cancer in mice, we previously generated a novel mouse model that combines activated ErbB2 (MMTV-ErbB2 allele (15)) with the mutp53 allele R172H corresponding to human hotspot mutp53 allele R175H (12). We found that mutp53 substantially accelerates Her2-driven mammary tumorigenesis (15). The underlying molecular mechanism is a mutp53-driven oncogenic feed-forward loop governing a superior survival of cancer cells. We found that mutp53, through enhanced recycling and/or stability of ErbB2/EGFR, augments MAPK and PI3K signaling, leading to transcriptional phospho-activation of HSF1 at Ser326. Furthermore, mutp53 directly interacts with phospho-activated HSF1 and facilitates its binding to DNA-response elements, thereby stimulating transcription of HSPs (5). In turn, heat shock proteins more potently stabilize their oncogenic clients ErbB2, EGFR, mutp53, HSF1 (and, possibly, other oncogenes), thus, reinforcing tumor development (5). Consistently, we found that ErbB2 inhibition by lapatinib not only suppresses tumor progression, but does so, at least in part, via inactivation of HSF1 (16). On the other hand, the interception of the ErbB2-HSF1-mutp53 feed-forward loop by lapatinib destabilizes mutp53 protein in Hsp90- and Mdm2-dependent manner (4). Since mutp53 ablation, by genetic and pharmacological approaches, has been shown to have therapeutic effects *in vivo* (17), it is possible that mutp53 destabilization by lapatinib contributes to its anti-cancer activity.

In the present study, we identified HSF1 as a crucial upstream node responsible, at least in part, for the kinome adaptation of lapatinib-resistant cells *in vitro* and *in vivo*. We found that lapatinib-resistant cancer cells have enhanced HSF1 activity, a superior resistance to proteotoxic stress, and also lose their ability to degrade mutp53 in response to lapatinib. In contrast, HSF1 inhibition blocks lapatinib-induced kinome adaption and prevents the development of lapatinib resistance. Our data suggest a mechanism-based rationale for the clinical utilization of HSF1 inhibitors for the treatment of lapatinib-resistant Her2-positive breast cancer and/or – in combination with lapatinib - to prevent development of lapatinib resistance.

MATERIALS AND METHODS

Human cancer cells. Human Her2-positive breast cancer cell line BT474 carrying E285K *TP53* mutation was purchased from ATCC in 2013. ATCC verifies cell's identity with short tandem repeat analysis. To generate lapatinib-resistant BT474R cell line, parental BT474 cells were cultivated in the presence of increasing concentrations (100-300 nM) of lapatinib for 6 months, as previously described (3). No further cell's identity verification was performed. Unless indicated otherwise, lapatinib-resistant BT474R cells were routinely maintained in the presence of 300nM lapatinib. Where shown, cells were treated with indicated concentrations of lapatinib (L-4899, LC lab), MG132 (M7449, Sigma), ganetespib (STA-9090, Synta Pharmaceuticals, Lexington, MA, USA), KRIBB11 (385570, Calbiochem, Billerica, MA, USA). All cell viability assays were done using standard clonogenicity assays and CellTiter-Blue Cell Viability Assay (Promega, 96-well format with 5,000 cells/well seeded 24 hrs prior). Prior to the CTB assay (Fig. 1A), cells were maintained in lapatinib-free media for 3 days. Cells were treated with drugs for 48 hours, unless indicated otherwise, with drug concentrations as shown. Florescence was detected by SPECTRAmax M2 (Molecular Devices).

Animals. MMTV-ErbB2 mice harboring activated ErbB2 were from Jackson Labs (strain FVBN-Tg(MMTV-ErbB2)NK1Mul/J). mutp53 R172H mice were a gift from G. Lozano (27). Generation of R172H/+;ErbB2 compound mice was described previously (15). 8 weeks old R172H/+;ErbB2 littermate females, all on C57Bl6/J:FVB/N 50:50 background, were treated with vehicle (18% Cremophor/3.6% dextrose) or lapatinib (75 mg/kg three times a week) by oral gavage life-long. When lapatinib-treated tumors acquired lapatinib resistance, animals were treated with either vehicle, lapatinib alone or lapatinib with ganetespib, as described in the text. Ganetespib was prepared as previously described (17) and injected into the tail vein at 50 mg/kg once a week. At endpoint (tumor size $\sim 3.5 \text{ cm}^3$) mice were sacrificed and some of lapatinib only treated tumors were used to establish cell cultures. Mice were treated according to the guidelines approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC).

Establishing primary mammary tumor cell cultures. Mammary tumors were dissected from mice, rinsed three times in PBS and sequentially digested with collagenase/hyaluronidase (37°C, 2h), 0.05% Trypsin, DNase I and Dispase (Stem Cell Technology). The ensuing cell suspensions were treated with red blood cell lysis buffer, rinsed with PBS, resuspended in Opti-MEM medium (Gibco) and passed through a 40 μm mesh to remove cell chunks. Cells were plated on gelatin-coated plates and grown in CnT-BM1 medium (Cell-N-Tec). Unless indicated

otherwise, lapatinib-resistant 125R cells derived from a lapatinib-resistant mammary tumor were routinely maintained in the presence of 300nM of lapatinib. Heterozygous mutant p53 R172H/+ status was verified and confirmed by using genotyping primers (27) in all established mouse cell lines.

Immunoblot analysis and kinome arrays. For immunoblots, cell lysates with equal total protein content (2-20µg) were blotted with antibodies to p53 (FL393), Mdm2, GAPDH, Hsc70 (all from Santa Cruz Biotechnology); Erk1, pErk1/2 (T202/Y204), EGFR, pEGFR (Y845), ErbB2, pErbB2 (Y1221/1222 and pY1248), MET, cleaved PARP, PDGFR α , PDGFR β , FGFR, AKT, pAKT MdmX, pTK (all from Cell Signaling); HSF1, pHSF1 (S326), Hsp90, Hsp70, Hsp27 (all from Enzo Life Sciences Inc, Farmingdale, NY). The phospho-RTK array on primary mammary tumor cells was performed according to the manufacturer's protocol (Mouse Phospho-RTK Array Kit, R&D Systems).

Statistical analysis. Unpaired two-tailed Student's *t*-test was used to compare tumor measurements. Kaplan-Meier analysis and log rank statistics was used to compare animal survival.

RESULTS

Generation and characterization of human and mouse lapatinib-resistant ErbB2-positive breast cancer cell lines

To gain the mechanistic insight into lapatinib resistance we utilized two complementary approaches: *in vitro* and *in vivo*. For *in vitro* studies, we continuously cultivated human Her2-positive BT474 breast cancer cells in the presence of increasing concentrations (100-300 nM) of lapatinib for 6 months. All selected lapatinib-resistant clones were combined and maintained as a pool, as previously described (3). Lapatinib-resistant cells approximately doubled their viability compared to lapatinib-sensitive cells (Fig. 1A), which was associated with decreased apoptosis in the presence of lapatinib (Fig. 1B).

To establish and investigate an *in vivo* model of lapatinib resistance, we utilized the previously described MMTV-ErbB2;R172H mouse model of Her2-positive breast cancer ('R172H/+;ErbB2' hereafter) (15). p53 R172H mutation in mice corresponds to p53 R175H mutation in humans, a hot-spot in sporadic Her2-positive breast cancer (12). At the age of mammary micro-lesions (8 weeks old), R172H/+;ErbB2 females were given lapatinib (75 mg/kg three times a week) or vehicle by oral gavage, life-long. Consistently with human data, lapatinib shows a tendency to delay tumor onset (from 256 to 319 days, median onset, $p=0.091$) and significantly extended overall survival (from 321 to 362 days, median survival, $p=0.014$) compared to vehicle-treated mice (Fig. 1C). Similarly, to human clinical data, after initial response (Fig. 1C) mouse mammary tumors acquired lapatinib resistance and started to exhibit growth kinetics similar to vehicle-treated tumors (Fig. 4A).

We established cell lines from both vehicle-treated (i.e. lapatinib-sensitive; 1349, 1347, 1251, 1252, 1253) and lapatinib-treated (i.e. lapatinib-resistant; 125R) mouse mammary tumors. In contrast to previous studies using human Her2-positive breast cancer cell lines that were derived from unrelated patients, have different p53 mutations (3) and acquired lapatinib resistance under more artificial *in vitro* conditions, our murine cell lines were derived from littermates, have an identical genetic background (see Methods), the same p53 mutational status (R172H) and - most importantly - acquired lapatinib resistance *in vivo* (with normal gland architecture, tumor microenvironment, immune system status etc.) and therefore should better reflect the resistance mechanisms encountered in patients in the clinic. The short-term cell viability assay and the long-term colony formation assays both confirmed that the established

cell lines continued to maintain their lapatinib resistance acquired *in vivo* (Fig. 1D, E).

To test for possible compensatory mechanisms in lapatinib-resistant compared to lapatinib-sensitive R172H/+;ErbB2 primary mammary tumors, we performed the kinome profiling of 39 activated RTKs (Mouse-specific Phospho-RTK Array Kit) in lapatinib-treated vs. vehicle-treated tumors, respectively. As expected, we found downregulation of phospho-activated ErbB2 and EGFR and upregulation of multiple compensatory RTKs (Fig. 1F), including previously described Axl (2) and novel RTKs, such as NFGFR, MUSK, VEGFR1, PDGFR α , PDGFR β , EPHA2 and EPHB2 (Fig. 1F). These results suggest an extensive kinome reprogramming and a switch to multiple alternative RTKs instead of the ErbB2 pathway in lapatinib-resistant cells. Consistently, we observed enhanced phospho-Erk, a common downstream RTK effector, in lapatinib-resistant 125R murine cell line (Fig. 1G). We validated the results of *in vivo* kinome arrays by Western blot analysis on the cell lines established *in vitro* from murine mammary tumors. Consistently with the array, PDGFR α and PDGFR β were upregulated in lapatinib-resistant 125R cells compared to lapatinib-sensitive cells (Fig. 1G). Interestingly, in human lapatinib-resistant BT474 PDGFR α and PDGFR β were not upregulated and instead, MET was elevated (Fig. 1H). Our results are consistent with previously described lapatinib-resistant BT474 cells that were independently generated and characterized (3). In agreement with this study, we found that lapatinib-resistant BT474 have downregulated ErbB2 signaling and instead, rely on other RTK pathways. Surprisingly, this is not observed in mouse tumor cells that have acquired lapatinib resistance *in vivo*. This difference likely reflects the heterogeneity in adaptive responses noted previously (3).

Despite the distinct adaptive RTK response in mouse vs. human lapatinib-resistant cancer cells, notably, they share an important common feature, i.e. stabilized PDGFR α , PDGFR β and MET that are maintained by heat shock proteins

(<https://www.picard.ch/downloads/Hsp90interactors.pdf>). Therefore, we hypothesized that HSF1-mediated heat shock response is causative to the observed adaptive RTKs upregulation in lapatinib-resistant cells. Indeed, this link is supported by the fact that six out of eight RTKs upregulated in lapatinib-resistant mammary tumors - Axl, VEGFR1, MUSK, PDGFR β , PDGFR α , EPHA2 - are known Hsp90 clients (www.picard.ch/downloads/Hsp90interactors.pdf).

Lapatinib-resistant breast cancer cells are resistant to proteotoxic stress

To test whether HSF1-induced heat shock response is involved in the kinome adaptation of lapatinib-resistant cells, we compared their viability under the proteotoxic stress condition with lapatinib-sensitive cells. We found both, the cells that acquired lapatinib resistance *in vitro* (Fig. 2A) and *in vivo* (Fig. 2B) to be more resistant to the proteotoxic stress induced by the proteasome inhibitor MG132, which correlated with reduced apoptosis measured by PARP cleavage (Fig. 2C).

HSF1 reveals its protective role under proteotoxic stress via transcriptional activation of HSPs by transcriptionally active pSer326-HSF1. Indeed, upon proteotoxic stress lapatinib-resistant BT474 cells show a higher level of pSer326-HSF1 (Fig. 2D). Since pSer326-HSF1 antibodies are human-specific, we tested activity of pHSF1 in murine lapatinib-resistant 125R cells by the level of pHSF1 transcriptional targets Hsp70 and Hsp90, and again found their significant upregulation upon proteotoxic stress (Fig. 2E). These data indicate that lapatinib resistance correlates with augmented HSF1 function and, as a result, with a superior tolerance to proteotoxic stress.

Lapatinib fails to modulate the ErbB2-HSF1-mutp53 axis in lapatinib-resistant breast cancer cells

We previously described a novel oncogenic ErbB2-HSF1-mutp53 feed-forward loop leading to HSPs-mediated stabilization of ErbB2 and mutp53 (5,15). Interception of this circuit by lapatinib downregulates activation of HSF1 and its target Hsp90 and releases the E3 ubiquitin ligase Mdm2 from an inhibitory complex with Hsp90, leading to destabilization of mutp53 (4). We now tested the effect of lapatinib on mutp53 levels in lapatinib-resistant BT474 cells (Fig. 3A) and found that lapatinib lost its ability to destabilize mutp53 even at higher doses (Fig. 3A), likely as a result of chronic HSF1 activity. Indeed, lapatinib did not suppress HSF1 or its transcriptional target Hsp70, compared to lapatinib-sensitive cells, and failed to induce HSP-mediated auto-degradation of Mdm2 and its *bona fide* substrates MdmX and mutp53 (Fig. 3A). Since previous studies identified highly stabilized mutp53 protein as an essential pro-survival factor in cancer cells (17), mutp53 depletion by lapatinib in lapatinib-sensitive cells could further enhance lapatinib's efficiency, while unresponsive high levels of mutp53 in lapatinib-resistant cells might contribute to the resistance mechanism. Similarly, lapatinib inhibited ErbB2 signaling (measured

by phospho-ErbB2) and Hsp70 levels in sensitive murine lines, but failed to do so in the lapatinib-resistant murine 125R cells (Fig. 3B). Furthermore, lapatinib blocks HSF1 activation by Ser326 phosphorylation in lapatinib-sensitive, but not in lapatinib-resistant BT474 cells after heat shock (42C, 30min) (Fig. 3C). Most likely, HSF1 lost its dependency on the ErbB2 signaling in lapatinib-resistant cells due to the switch to alternative RTKs and their downstream effectors like Erk and Akt (18,19), which reconstitutes HSF1 function and supports cells survival after ErbB2 inhibition.

Altogether, these data reinforce that despite of the heterogeneity of adaptive responses, tumors acquire lapatinib resistance, at least in part, via unified HSF1 guided mechanism that feeds into stabilization of mutp53.

Lapatinib-resistant breast cancer cells are sensitive to Hsp90 inhibition

Since the majority of adaptive RTKs that we identified *in vivo* (Fig. 1F) are known Hsp90 clients, we hypothesized that lapatinib-resistant cells retain sensitivity to Hsp90 inhibitors. Hsp90 inhibition is expected to simultaneously target not only identified RTKs, but also other relevant oncogenic Hsp90 clients, e.g. ErbB2, EGFR, HSF1 and mutp53 (20), and to produce a superior therapeutic response specifically in mutp53 expressing cancer cells. To test this hypothesis, we used ganetespib, a new generation Hsp90 inhibitor, which is currently in several clinical trials with a favorable safety profile (21). First, we tested the effect of ganetespib *in vivo*, using R172H/+;ErbB2 mice with mammary tumors that have been previously treated with lapatinib until they acquired resistance, i.e. lapatinib no longer suppressed their growth. Starting with the same average tumor size in each group, we designated three groups of animals (Fig. 4A): (i) animals previously treated with vehicle were continued on vehicle (Veh/Veh); (ii) animals previously treated with lapatinib (i.e. lapatinib-resistant) were continued on lapatinib alone (75 mg/kg three times a week lifelong) (Lap/Lap); (iii) some animals previously treated with lapatinib (i.e. lapatinib-resistant) were continued on lapatinib (75 mg/kg three times a week lifelong) together with ganetespib (50 mg/kg once a week lifelong) (Lap/Lap+Gan).

Consistently with their lapatinib resistance, the tumors on lapatinib alone continued to grow fast, with the rate very similar to vehicle-treated tumors (Fig. 4A, solid vs. small-dash lines). In contrast, addition of ganetespib significantly suppressed growth of lapatinib-resistant tumors

(Fig. 4A, wide-dash vs. small-dash lines). These data demonstrate that, despite lapatinib and ganetespib having overlapping targets (ErbB2, EGFR, mutp53), ganetespib overcomes lapatinib-resistant adaptive responses and efficiently curbs growth of lapatinib-resistant tumors *in vivo*. Consistently with these *in vivo* data, both human and mouse lapatinib-resistant cell lines were highly sensitive to ganetespib *in vitro* (Fig. 4B, C). As expected, ganetespib effectively inhibited ErbB2 signaling (measured by phospho-ErbB2) and - contrary to lapatinib - depleted mutp53 and Mdm2 in both lapatinib-sensitive and lapatinib-resistant cells (Fig. 4D). We speculate that ganetespib suppresses growth of lapatinib-resistant tumors via two complementary mechanisms: targeting of compensatory RTKs and release Mdm2 from the Hsp90 inhibitory complex, leading to mutp53 degradation.

HSF1 inhibition targets mutp53 and ErbB2 for degradation and suppresses growth of lapatinib-resistant breast cancer cells

Although Hsp90 inhibition seems to be an effective strategy to overcome lapatinib resistance, it has significant limitations. Hsp90 inhibitors have been shown to activate HSF1-mediated heat shock response, which in the long run protects cancer cells from apoptosis (7).

Therefore, the efficacy of Hsp90 inhibitors is limited by HSF1 function. Thus, we set to test the effect of specific HSF1 inhibitor KRIBB11 (N2-(1H-indazole-5-yl)-N6-methyl-3-nitropyridine-2,6-diamine) (22) on lapatinib-resistant vs. lapatinib-sensitive cells. Consistently with a previous report (22), KRIBB11 inhibits HSF1 phosphorylation with or without proteotoxic stress (MG132) (Fig. 5A). As a readout of HSP suppression, KRIBB11 also dose-dependently suppressed Hsp90 clients ErbB2, mutp53 and Mdm2 in both lapatinib-sensitive and lapatinib-resistant human BT474 and mouse 125R cancer cells (Fig. 5B, C). Similarly, to Hsp90 inhibition by ganetespib (Fig. 4D), KRIBB11 reactivated Mdm2 E3 ligase activity as manifested by downregulation of Mdm2 ubiquitination substrates MdmX, mutp53 and Mdm2 itself (Fig. 5B), which was rescued by the proteasome inhibitor MG132 (Fig. 5A, lanes 3, 4, Fig. 5D). These data indicate that HSF1 inhibition by KRIBB11 simultaneously targets both key oncogenic drivers, ErbB2 and mutp53, in lapatinib-sensitive and lapatinib-resistant ErbB2-overexpressing breast cancer cells. As a result, KRIBB11 dose-dependently kills lapatinib-sensitive and lapatinib-resistant human (Fig. 5E) and mouse (Fig. 5F) breast cancer cells with comparable efficiency.

HSF1 inhibition suppresses adaptive RTK activation and overcome lapatinib resistance in Her2-positive breast cancer cells

Consistent with previous studies (3), we noted a substantial heterogeneity of adaptive responses in lapatinib-resistant cancer cells, including RTKs such as MET (3) and PDGFR α in human and mouse cells, respectively (Fig. 1). Interestingly, activation in response to lapatinib of both, MET (Fig. 6A, B) and PDGFR α (Fig. 6C) occurred as quickly as 48 hrs after lapatinib treatment in lapatinib-resistant, as well as lapatinib-sensitive cells. It appears that it takes place at posttranscriptional level. RNAseq analysis of BT474 cells treated with lapatinib did not reveal induction of MET RNA transcript, while MET signaling was shown to be activated (3). Since MET (23) and PDGFR α (24) are both Hsp90 clients, we asked if HSF1 inhibition by KRIBB11 would reverse MET and PDGFR α lapatinib-induced compensatory upregulation. Indeed, even the low KRIBB11 dose (1 μ M, compare to Fig. 5B, E) alleviated lapatinib-induced MET upregulation in both lapatinib-sensitive and lapatinib-resistant BT474 cells (Fig. 6A, B). Moreover, KRIBB11 synergized with lapatinib in degrading mutp53 and EGFR in lapatinib-sensitive BT474 cells (Fig. 6A) and restored mutp53 responsiveness to lapatinib in lapatinib-resistant BT474R cells (Fig. 6B).

In addition, global assessment of Tyr-phosphorylated proteins – as an indirect readout of overall levels of kinases - revealed an extensive and dose-dependent kinome activation in response to lapatinib in murine 125R cells (Fig. 6D, *left*), while HSF1 inhibition by KRIBB11 suppressed global kinome activation (Fig. 6D, *right*) and individual Hsp90 kinase clients, e.g. ErbB2/pErbB2 (Fig. 5C), FGFR (Fig. 6E). Similarly, global pTK signal and individual Hsp90 client kinases, e.g. ErbB2, pErbB2 (Fig. 5B), FGFR, MET, EGFR, AKT and pAKT (Figs. 5B, 6F), were downregulated in human BT474 cells in response to HSF1 inhibition in a dose dependent manner.

Finally, we asked whether HSF1 inhibition by KRIBB11 would affect formation of lapatinib-resistant clones in a long-term colony formation assay. We found that while lapatinib-sensitive murine 1349, 1347 and 1253 cells treated with lapatinib did develop resistant clones, as did cells treated with KRIBB11 alone, the combinatorial lapatinib/KRIBB11 treatment completely blocked growth of lapatinib-resistant colonies (Fig. 6G). Taken together, these results indicate that HSF1 inhibition suppresses global activation of compensatory RTK pathways in response to lapatinib and, therefore, can prevent the onset of lapatinib resistance.

DISCUSSION

Although ErbB2-targeted therapies, such as lapatinib, revolutionized ErbB2-overexpressing breast cancer management, primary and acquired resistance remains a major obstacle for the cure of this deadly disease. Therefore, understanding the mechanisms of lapatinib resistance will greatly facilitate development of successful combinatorial treatments with a durable therapeutic effect. Tissue culture experiments using cancer cells that acquired lapatinib resistance *in vitro* identified a number of compensatory pathways activated in response to ErbB2 inhibition (2). However, the therapeutic utility of these findings remains unclear and has not been validated in the clinic thus far. In this study, we for utilized an preclinical MMTV-ErbB2;mutp53 mouse model to investigate the lapatinib resistance mechanisms acquired *in vivo* in Her2-positive mammary tumors and to compare them to the resistance mechanisms acquired *in vitro*.

In both *in vivo* and *in vitro* scenarios, we found a kinome re-organization in response to ErbB2 inhibition by lapatinib. In agreement with previous studies (3), a substantial heterogeneity of adaptive responses was observed in lapatinib-resistant cancer cells, including previously described (Axl, MET) (2) and novel upregulated pathways, such as NFGR, MUSK, VEGFR1, PDGFR α , PDGFR β , EPHA2 and EPHB2 (Fig. 1F). This multifaceted nature of compensatory responses underscores the difficulty of choosing the most effective drug combination to prevent or overcome lapatinib resistance. In this study we uncovered a common pro-survival mechanism of lapatinib resistance acquired *in vivo* and *in vitro*, i.e. an augmented HSF1-mediated heat shock response.

The oncogenic cooperation between ErbB2 and HSF1 was noted previously. Several *in vivo* studies demonstrated a crucial role of HSF1 in the development of ErbB2-driven breast cancer (10). Thus, genetic ablation of HSF1 significantly improves survival of MMTV-Neu mice (10) and HSF1 transcriptional targets Hsp90 (11), Hsp70 (11) and Hsp27 (7) have been shown to stabilize ErbB2. Not surprisingly, HSF1 protein levels are elevated in 80% of breast cancer cases, leading to enhanced expression of its targets such as Hsp90, and high levels of HSF1 are associated with poor prognosis (25). Although no clinical studies have directly analyzed the levels of HSF1 or HSPs in lapatinib-resistant breast cancer (or any other Her2-overexpressing cancer), emerging clinical evidence strongly supports our main conclusion. Thus, Phase II clinical trial with an Hsp90 inhibitor tanespimycin (aka 17-AAG) plus trastuzumab (Her2-targeted

therapy) showed a significant anticancer activity in patients with Her2-positive trastuzumab-resistant metastatic breast cancer (26). Altogether, these data strongly support the idea that HSF1 is a promising therapeutic target, especially in the context of resistance to Her2-targeted therapies.

Furthermore, we previously demonstrated that HSF1 is an important downstream effector of ErbB2 signaling and that lapatinib inhibits transcriptional activation of HSF1, by suppressing its Ser326 phosphorylation (5). Most likely, lapatinib affects HSF1 function by inhibiting MAPK and AKT activation, both of which can induce transcriptional phospho-activation of HSF1 at Ser326 (18,19). On the other hand, upregulation of compensatory RTKs in lapatinib-resistant cells can induce sustained MAPK and AKT signaling leading to enhanced S326-HSF1 phosphorylation and HSF1 protein stability. In support of this hypothesis we observed higher HSF1 protein and S326-HSF1 level after heat shock in lapatinib-resistant cells (Fig. 1G, H, Fig. 2D).

Human Her2-positive breast cancers have a high 72% prevalence of mutp53 (12), which are predictive of poor prognosis due to a more aggressive disease and increased susceptibility to metastasis (12,14). In agreement with clinical data, we previously uncovered novel oncogenic activity of mutp53 – amplification of ErbB2 activity. We found that ErbB2 - via MAPK and PI3K pathways - potentiates Ser326 phospho-activation of HSF1, while mutp53 directly interacts with and facilitates the binding of Ser326 phospho-HSF1 to its DNA response elements promoting HSPs expression (4,15). On contrary, ErbB2 inhibition in lapatinib-sensitive cells impedes HSF1 activation, leading to the release of Mdm2 from its inhibitory complex with Hsp90 and to mutp53 destabilization (4) (Fig. 3A; schematic diagram in Fig. 6H). Strikingly, we now found that in lapatinib-resistant cells, lapatinib fails to modulate the ErbB2-HSF1-mutp53 axis (Fig. 3). Instead, HSF1 is constitutively activated and does not depend on the ErbB2 signaling (Fig. 3C), resulting in a superior tolerance of lapatinib-resistant cells to proteotoxic stress (Fig. 2). It appears that in response to ErbB2 inhibition, lapatinib-resistant cancer cells hijack the heat shock response for their survival.

We speculate that in lapatinib-resistant cells, the HSF1 function is restored by activation of adaptive RTKs and their downstream signaling components (Fig. 6H). In turn, sustained expression of HSPs promotes stability of their clients, adaptive RTKs, thus maintaining continuous HSF1 function (Fig. 6H). Although some elements of this model await further investigation, here we identified HSF1 as a central node of the lapatinib resistance mechanisms

and demonstrated that its inhibition (i) suppresses global tyrosine-phosphorylation (Fig. 6D, F), (ii) alleviates lapatinib-induced upregulation of specific adaptive RTKs (Fig. 6A, B, C), (iii) synergizes with lapatinib in degradation of mutp53 (Fig. 6A, B) and (iv) prevents development of lapatinib resistance, as measured by appearance of lapatinib-resistant colonies (Fig. 6G).

Importantly, HSF1 inhibition in lapatinib-resistant cells restores mutp53 destabilization in response to lapatinib (Fig. 6B). Highly stabilized mutp53 levels are required for mutp53 oncogenic gain-of-function (17), and mutp53 genetic and pharmacological ablation significantly suppresses malignant phenotypes in mutp53-carrying cancers (17). Therefore, identification of compounds targeting mutp53 for degradation has a major translational impact for Her2-positive breast cancer therapy, given the high frequency of p53 mutations in this breast cancer subtype.

It still needs to be explored whether mutp53 unresponsiveness to lapatinib contributes to lapatinib resistance and whether KRIBB11 cytotoxic effects are - at least in part - mediated by mutp53 destabilization. In support of latter link, we previously reported a strong correlation between high levels of mutp53 and nuclear Ser326 phospho-HSF1 in 150 human breast cancer biopsies, exclusively in Her2-positive, but not in Her2-negative;ER/PR-positive breast cancer samples (5).

Our study is highly translationally relevant, because it showed that pharmacological inhibition of HSF1 simultaneously inhibits diverse adaptive responses endowing lapatinib resistance, as well as destabilizes potent oncogenic drivers of Her2-positive breast cancer, such as ErbB2, EGFR and mutp53. While KRIBB11 has not been tested *in vivo* or in a clinic, a novel drug with anti-HSF1 activity curaxin (CBL0137, Incuron) recently entered Phase 1 clinical trials for metastatic or unresectable advanced solid cancers and lymphomas.

Our study proposes HSF1 as a new target to prevent and/or delay onset of lapatinib resistance and opens up a new therapeutic possibility for the clinical application of HSF1 inhibitors with a potential of the instant clinical translation.

CONTRIBUTIONS

Conception and design: Marchenko N.

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Figure Legends

Figure 1. Generation and characterization of lapatinib-resistant human and mouse Her2-positive cancer cell lines

(A, B) Lapatinib-resistant human BT474R cells exhibit a two-fold increased viability after 48 hr treatment with lapatinib (A) and a significantly decreased apoptosis after treatment with 300nM lapatinib for indicated times (measured by cleaved PARP, B) compared to lapatinib-sensitive parental BT474 cells. (B) Western blot analysis, GAPDH is a loading control.

(C) Tumor onset is significantly delayed in R172H/+;ErbB2 females treated with 75 mg/kg lapatinib three times a week starting at 8 wks of age lifelong (red line) compared to vehicle-treated siblings (black line). Kaplan-Meier analysis, log rank statistics.

(D, E) Murine primary cell lines established from lapatinib-sensitive mammary tumors (1252, 1253, 1349) and from a lapatinib-resistant mammary tumor (125R) maintain lapatinib sensitivity and lapatinib resistance *in vitro*, respectively. (D) Short-term cell viability assay (48 hrs). (E) Long-term colony formation assay (4 wks).

(F) Lapatinib induces activation of multiple adaptive RTKs *in vivo*. Representative images of the mouse Phospho-RTK array kit comparing lapatinib-treated (i.e. lapatinib-resistant, top) with vehicle-treated (i.e. lapatinib-sensitive, bottom) tumors. 1. EGFR*, 2. ErbB2*, 3. ErbB3, 4. PDGFR α *, 5. PDGFR β *, 6. Axl*, 7. NGFR, 8. VEGFR1*, 9. MUSK*, 10. EPHA2*, 11. EPHB2. Known Hsp90 clients are marked with an asterisk. Triangles mark reference spots.

(G, H) Murine lapatinib-resistant 125R cells show upregulated PDGFR α and PDGFR β compared to murine lapatinib-sensitive cells 1349,1251,1252,1253 (G), while human lapatinib-resistant BT474R cells have upregulated MET and down-regulated ErbB2 and EGFR signaling (measured by phospho-ErbB2 pY1221/2 and pY1248, two top panels), phospho-EGFR and their effector phospho-Erk), compared to parental BT474 cells (H). Note that both human and mouse lapatinib-resistant cells have upregulated HSF1 and its transcriptional targets Hsp90 (G) and Hsp70 (H). Western blot analysis, constitutive heat shock protein 70 (Hsc70) and GAPDH served as a loading control.

Figure 2. Lapatinib-resistant cells are protected from proteotoxic stress

(A, B) Lapatinib-resistant human BT474R cells (A) and mouse 125R cells (B) are more resistant to proteotoxic stress induced by the proteasome inhibitor MG132 (0.5 μ M for 48 hrs) than their corresponding lapatinib-sensitive control cells. Cell viability assay.

(C-E) Under proteotoxic stress induced by the proteasome inhibitor MG132, lapatinib-resistant human BT474R cells have decreased apoptosis (cleaved PARP, **C**) and increased phospho-HSF1 (Ser326, **D**) compared to lapatinib-sensitive parental BT474 cells, while lapatinib-resistant murine 125R cells have upregulated heat shock proteins Hsp70 and Hsp90 compared to lapatinib-sensitive cells 1252, 1253 (**E**). Western blot analysis. GAPDH and Hsc70 as a loading control.

Figure 3. Lapatinib fails to modulate the ErbB2-HSF1-mutp53 axis in lapatinib-resistant cells

(A, B) Downregulation of HSF1, mutp53 and Mdm2 in lapatinib-sensitive parental BT474, but not in lapatinib-resistant BT474R cells, treated with indicated lapatinib concentrations for 24 hrs (**A**) or with an even increased lapatinib dose and longer time (300nM up to 72 hrs, **B**). (**C**) Lapatinib (300nM, 48h) blocks ErbB2 activation (measured by phospho-ErbB2) and its downstream effector Hsp70 in murine lapatinib-sensitive (1251, 1252, 1253), but not in lapatinib-resistant 125R cells. Hsc70 served as a loading control. (**D**) Lapatinib (300nM, 24 hrs) blocks HSF1 activation (measured by phospho-Ser326) in human lapatinib-sensitive BT474, but not in lapatinib-resistant BT474R cells after heat shock (42°C, 30min). Cells were pre-treated with lapatinib followed by heat shock. Western blot analyses. GAPDH as a loading control.

Figure 4. Lapatinib-resistant breast cancer cells are sensitive to Hsp90 inhibition

(A) R172H/+;ErbB2 female mice were treated either with vehicle or 50 mg/kg lapatinib (three times a week starting at 8 wks of age) until tumors acquired lapatinib resistance, i.e. lapatinib no longer suppressed tumor growth. At this point, previously vehicle-treated mice were continued on vehicle (Veh/Veh), while previously lapatinib-treated mice continued to be treated with either lapatinib alone (Lap/Lap) or with lapatinib together with 50 mg/kg ganetespib once a week (Lap/Lap+Ganet), as described in Results. Note that the initial tumor size in all three groups was on average comparable. Tumor size was measured and plotted every five days. Note that while lapatinib-resistant tumors grew similarly to untreated tumors (did not respond to lapatinib), addition of ganetespib significantly suppressed tumor growth (wide-dash line). * $p < 0.05$, ** $p < 0.01$, Student's *t*-test. Top asterisks compare the Lap/Lap+Ganet group with Lap/Lap, bottom asterisks compare the Lap/Lap+Ganet group with the Veh/Veh group. (**B, C**) Lapatinib-sensitive human BT474 (**B**) and murine 125R (**C**) cells have similar sensitivity to ganetespib as their corresponding lapatinib-sensitive cells (BT474 and 1251, 1252, 1253,

respectively). Cells were treated with DMSO or 0.3 μ M ganetespib for 48h, followed by the cell viability assay.

(D) Ganetespib (indicated concentrations, 24 hrs) inhibits ErbB2 signaling (measured by phospho-ErbB2 and phospho-Erk) and destabilizes mutp53 and Mdm2 in both, lapatinib-sensitive BT474 and lapatinib-resistant BT474R cells. Western blot analysis, GAPDH is a loading control.

Figure 5. HSF1 inhibition causes degradation of mutp53 and ErbB2 and suppresses growth of both, lapatinib-sensitive and lapatinib-resistant cancer cells

(A-D) The HSF1 inhibitor KRIBB11 suppresses activation of HSF1 (measured by phospho-Ser326) after MG132-induced proteotoxic stress (1 μ M, 2.5 h) in lapatinib-sensitive BT474 cells (A), suppresses ErbB2 signaling and destabilizes mutp53 in both lapatinib-sensitive BT474 and lapatinib-resistant BT474R cells (B), suppresses ErbB2 signaling and HSF1 target Hsp27 in both lapatinib-sensitive 1349 and lapatinib-resistant 125R murine cells (C); and induces degradation of mutp53 and Mdm2 in lapatinib-sensitive BT474 cells, which is rescued by the proteasome inhibitor MG132 (D). Cells were pre-treated with KRIBB11 (2.5 μ M, 24 hrs) followed by MG132 treatment (1 μ M, 2.5 hrs) (A) or simultaneously treated with KRIBB11 (2.5 μ M) and MG132 (2.5 μ M) for 24 h (D). Western blot analyses, GAPDH and Hsc70 served as a loading control.

(E, F) The HSF1 inhibitor KRIBB11 suppresses growth of human BT474R (E) and mouse 125R (F) lapatinib-resistant cells as efficiently as their corresponding lapatinib-sensitive controls, BT474 and 1251, 1252, 1349 cells, respectively. Cells were treated with indicated concentrations of KRIBB11 for 48 hrs, followed by cell viability assays, which are shown relative to DMSO-treated cells.

Figure 6. HSF1 inhibition bypasses lapatinib-induced adaptive signaling and prevents the onset of lapatinib resistance

(A, B) While lapatinib (0.1 μ M, 48 hrs) upregulates MET in both lapatinib-sensitive BT474 (A) and lapatinib-resistant BT474R (B) cells, HSF1 inhibitor KRIBB11 reverses this effect. Moreover, KRIBB11 synergizes with lapatinib in degradation of EGFR and mutp53 (A) and restores responsiveness of mutp53 to lapatinib (B). Western blot analyses, GAPDH is a loading control.

(C-F) Lapatinib (at indicated concentrations, 48 hrs) induces PDGFR α in both lapatinib-sensitive 1251 and lapatinib-resistant 125R murine cells (C) and induces global kinome activation

(measured by phospho-Tyr antibody, pTK) in lapatinib-resistant 125R cells (**D**), KRIBB11 inhibits the global pTK activity and individual kinases in 125R cells (**D, E**) and BT474 cells (**F**). Western blot analysis, Hsc70 and GAPDH are loading controls.

(**G**) KRIBB11 cooperates with lapatinib (at indicated concentrations, for 4 weeks) in suppressing emergence of lapatinib-resistant colonies. Colony formation assay. Representative images out of two technical replicas.

(**H**) The proposed model. ErbB2 signaling mediates HSF1 activation (4, 16), which is potentiated by mutp53 via a feed-forward loop (5, 15), thereby upregulating Hsp90 clients including compensatory RTKs and mutp53 itself. Inhibition of ErbB2 by lapatinib leads to inhibition of HSF1 transcriptional activity and therefore decreased Hsp90 and release of Mdm2 from its inhibitory complex with Hsp90 (4, 5) and subsequent degradation of mutp53 and Mdm2. KRIBB11 simultaneously inhibits diverse adaptive RTKs, as well as destabilizes potent oncogenic drivers - ErbB2, EGFR and mutp53.

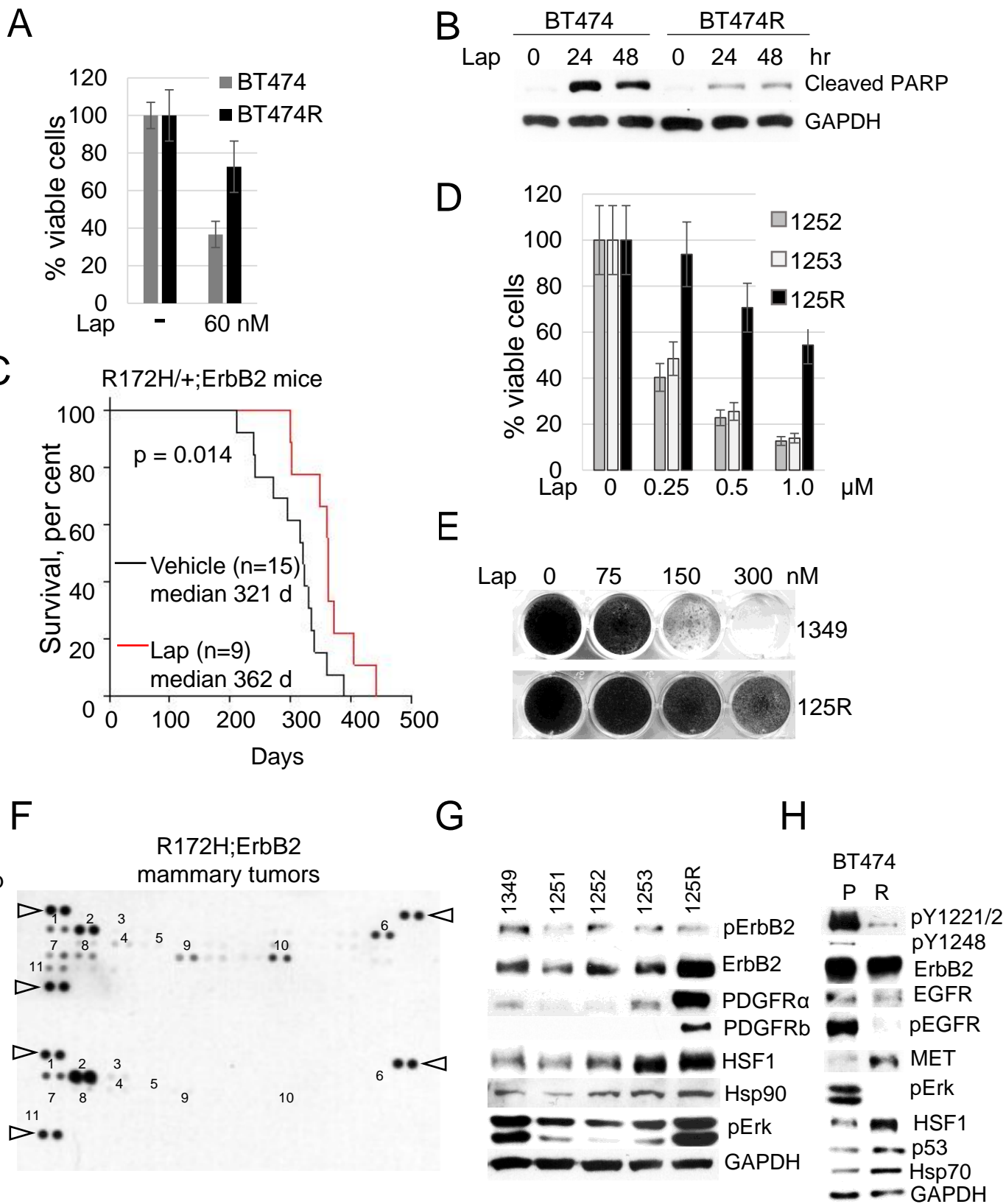


Figure 1

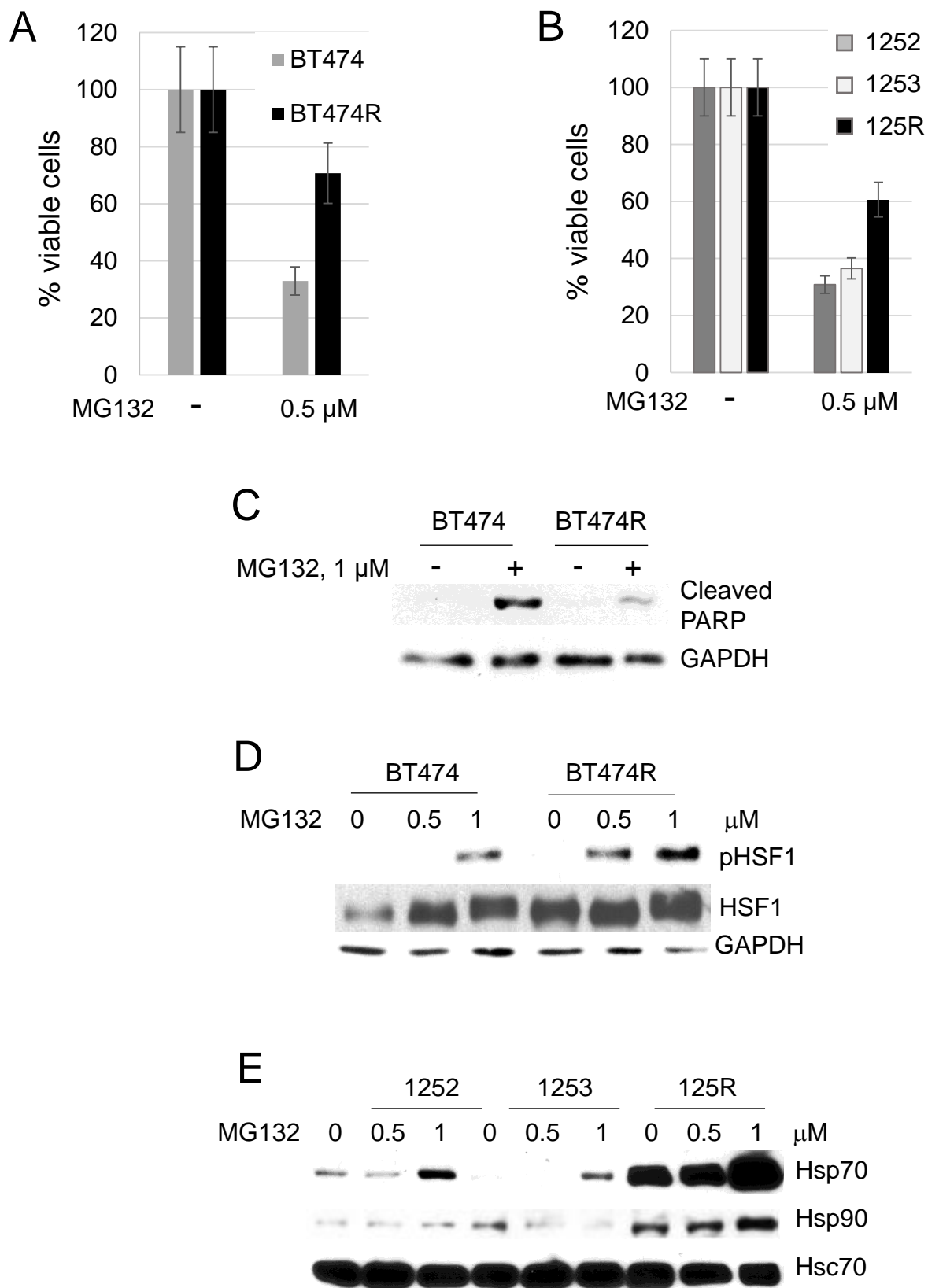
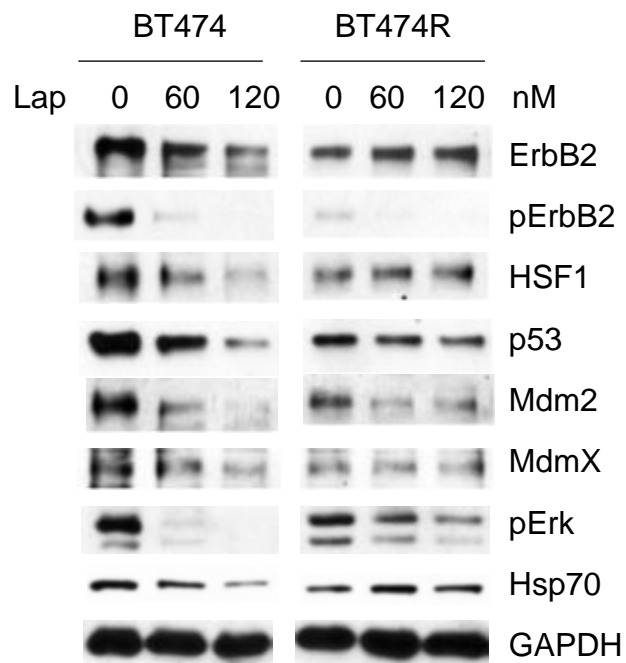
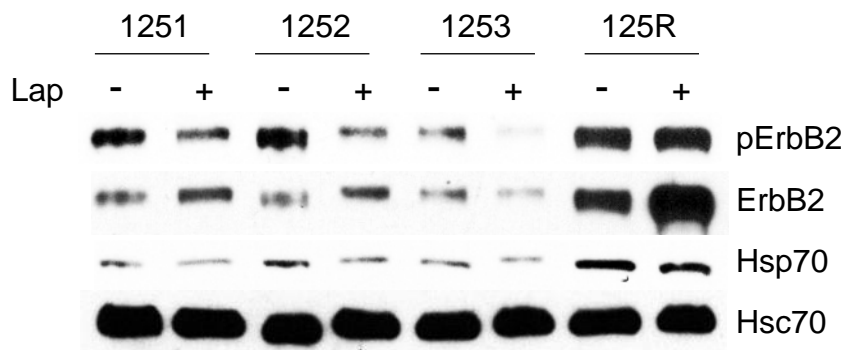


Figure 2

A



B



C

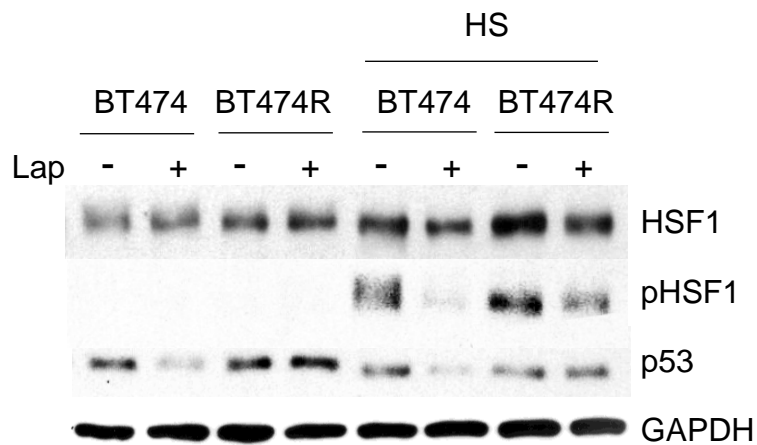


Figure 3

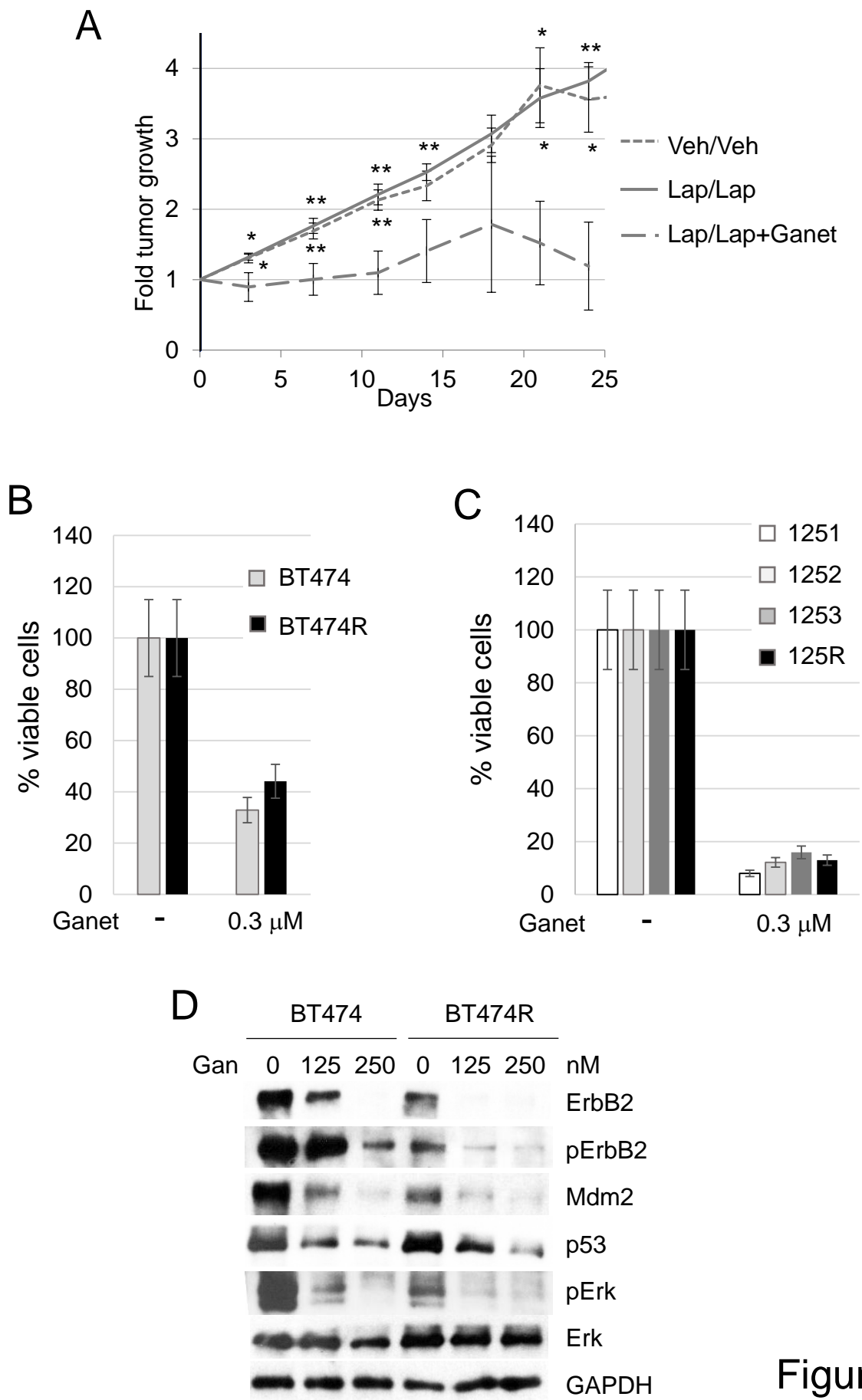


Figure 4

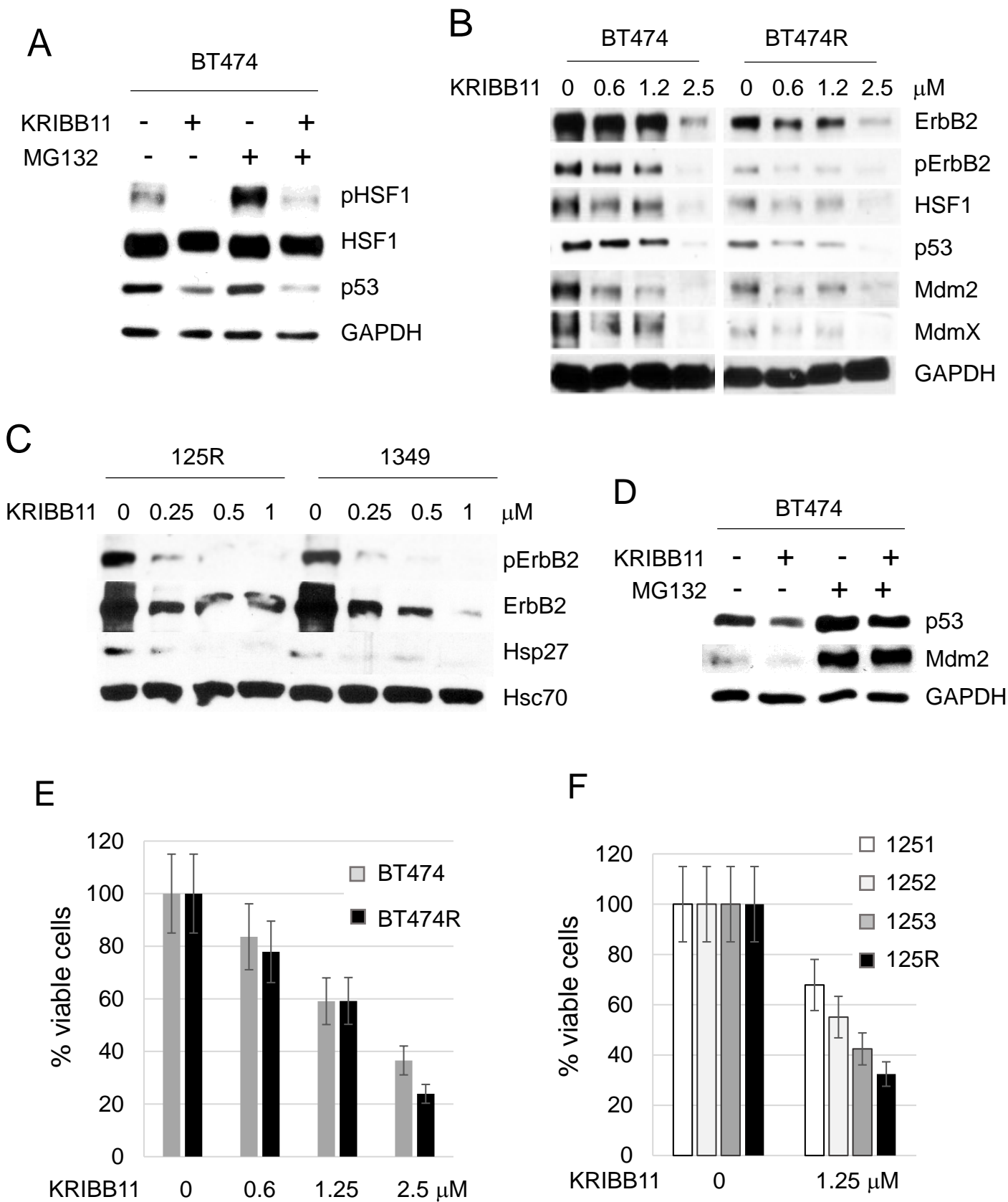


Figure 5

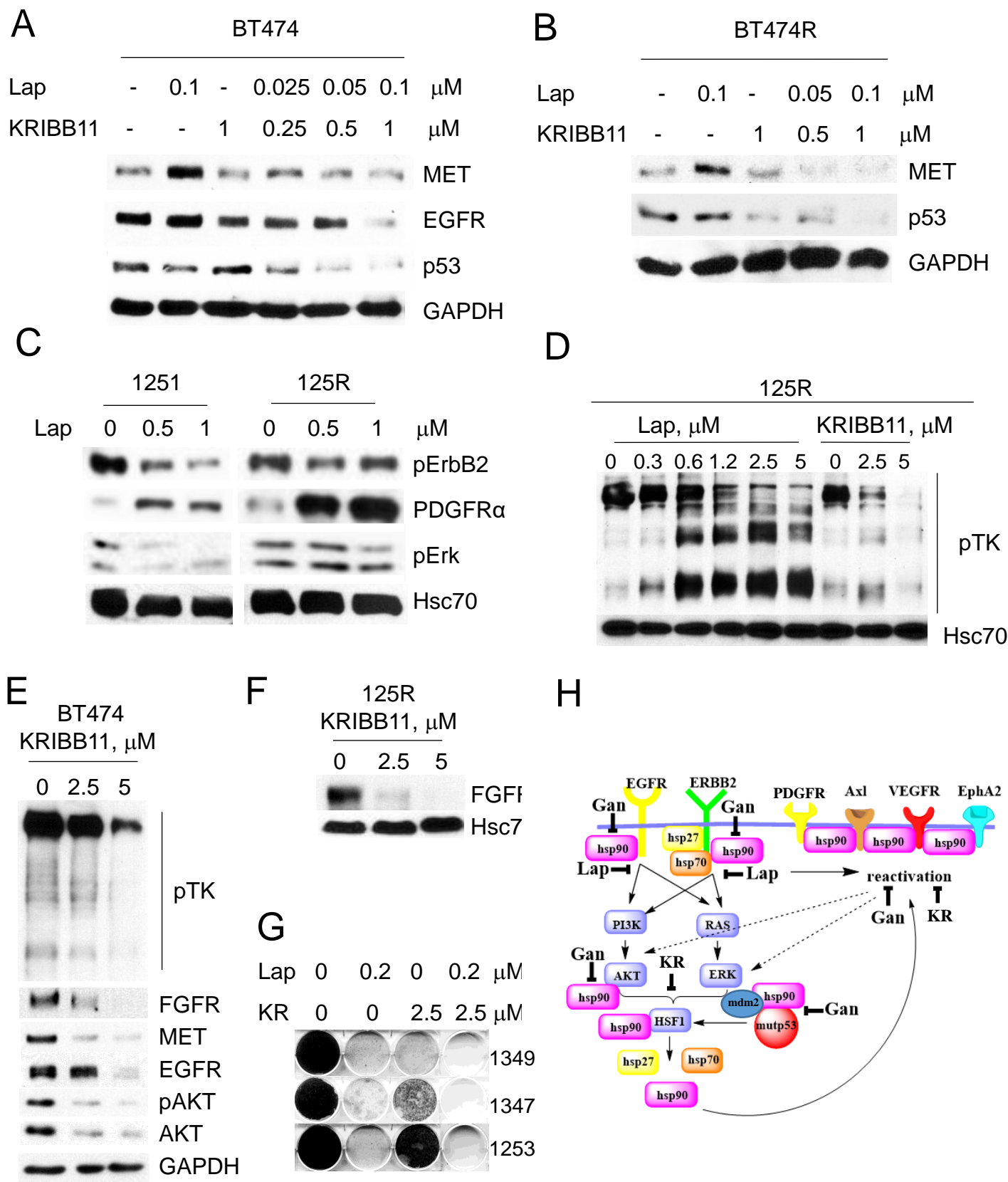


Figure 6